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(54) Title: METHODS FOR MEDIATING GENE SUPPRESION BY USING FACTORS THAT ENHANCE RNAi

(57) **Abstract:** The present invention is concerned with methods for enhancing gene suppression in cells and in particular it is concerned with improved methods for enhancing RNAi-mediated gene silencing by manipulation of factors associated with RNAi. The present invention is also concerned with methods for identifying factors which down-regulate as well as those which up-regulate RNAi. It is also concerned with genetic constructs useful for enhancing or modulating gene silencing and cells harbouring such constructs.

Methods for mediating gene suppression by using factors that enhance RNAi

TECHNICAL FIELD

The present invention is concerned with methods for enhancing gene suppression in cells and in particular it is concerned with improved methods for enhancing RNAi-mediated gene silencing by manipulation of factors associated with RNAi. The present invention is also concerned with methods for identifying factors which down-regulate as well as those which up-regulate RNAi. It is also concerned with genetic constructs useful for enhancing or modulating gene silencing and cells harbouring such constructs.

BACKGROUND OF THE INVENTION

The use of double-stranded RNA (dsRNA) to specifically interfere with gene expression has received considerable attention because of its demonstrated potency in a range of organisms, including some which have so far been genetically intractable. Termed RNA interference (RNAi), it has been implicated in viral defense, control of transpositional elements, genetic imprinting and endogenous gene regulation. It has been hypothesised to be the central mechanism in post-transcriptional gene silencing (PTGS), co-suppression, quelling, and antisense RNA-mediated gene suppression. One model that has been proposed is that dsRNA is fragmented into 21-25 nt species by dsRNA-specific nucleases, amplified by RNA-dependent RNA polymerase, and then dissociated and free to attack homologous mRNA by RNA nuclease-mediated degradation. The application of this technique will greatly facilitate the dissection of gene function and the validation of genes involved in disease states.

Recently at least two different strategies have been undertaken to identify the cellular proteins composing a proposed multi-protein complex involved in the recognition of dsRNA and the activation of dsRNA-mediated gene interference. The first involves the use of classical chemical mutagenesis or insertional mutagenesis to isolate mutants completely defective for RNAi and cloning of the relevant genes using complementation. These studies have been carried out in genetically tractable organisms such as plants, worms and fungi. The genetic screens described involve the use of RNAi systems in which the degree of suppression is complete. The mutagenesis produces mutants in which the RNAi effect is completely reversed indicating the loss of a cellular factor (function) required for the RNAi effect. Thus these genetic screens would most

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likely miss factors that have subtle effects or rate limiting or rate determining roles in RNAi.

The second strategy for finding key players in RNAi has involved the use of cell free assays. These in vitro reconstitution assays, on the other hand, identify cellular factors that impact on RNAi outside of the cellular context and therefore the cellular role of these factors must always be tested.

However, the major disadvantages of these strategies are that genes will not be identified if they are essential to the organism, nor will they directly identify gene activities which will enhance RNAi when overexpressed.

Thus there is a need for models which can demonstrate a range of RNAi efficacies, with both increasing and decreasing quantitative activities being selectable. This would enable the identification of factors which can enhance or reduce the gene silencing effect.

It is therefore an object of the present invention to overcome or at least ameliorate one or more disadvantages of the prior art, or provide a useful alternative.

SUMMARY OF THE INVENTION

Through the use of a fission yeast model for the study of dsRNA-mediated gene silencing and in the search for factors involved in this process, it was surprisingly found that the natural level of RNAi activity can be enhanced by manipulating factors associated with RNAi activity or efficacy. Thus, it has been found that by increasing the steady-state levels of a target nucleic acid sequence in the presence of the same pool of the corresponding antisense sequence, or a part thereof, the antisense-mediated suppression was not only maintained, but enhanced. This is indicative of an RNAi-like mechanism of gene suppression. It has also been found that overexpression of certain sequences, named herein *RNAi enhancing sequences (res)*, (also referred to herein as *a*nti-sense enhancing sequences – *aes*), also had the ability to enhance RNAi.

This ability of RNAi activity to be enhanced in *S. pombe* provides a model system which enables the analysis of RNAi processes and the identification and study of factors which either up-regulate or down-regulate its activity. The system has been further used to identify RNAi enhancing gene sequences which increase PTGS efficacy when their resulting protein activities are augmented in vivo. The model used to exemplify the present invention and the methods described are also applicable to

treatment of disorders in which gene expression requires more efficient modulation or silencing.

According to a first aspect there is provided a method for inhibiting the expression of a target nucleic acid in a cell, which method comprises the steps of

5   (i)   elevating in the cell the level of an RNAi factor, and

(ii)   prior, concurrently with or subsequent to performing step (i), introducing into the cell a molecule which is, or gives rise to, an anti-sense nucleic acid directed toward at least a portion of the RNA transcript of the target nucleic acid, under conditions permitting the RNAi factor to increase the degree to which the anti-sense nucleic acid

10   inhibits expression of the target nucleic acid.

According to a second aspect there is provided a method of increasing cellular susceptibility to anti-sense-mediated inhibition of target nucleic acid expression, which method comprises elevating the level of an RNAi factor in a cell that expresses said target nucleic acid, with the proviso that the cell is to have prior, concurrently or

15   subsequently introduced thereinto a molecule which is, or gives rise to, an anti-sense nucleic acid directed toward at least a portion of the RNA transcript of the target nucleic acid under conditions permitting the RNAi factor to increase the degree to which the anti-sense nucleic acid inhibits expression of the target nucleic acid.

According to a third aspect there is provided a method for treating a subject

20   suffering from a disorder whose alleviation is mediated by inhibiting the expression of a target nucleic acid, which method comprises the steps of

(i)   elevating the level of an RNAi factor in the subject's cells where the target nucleic acid is expressed, and

(ii)   prior, concurrently with or subsequent to performing step (i), introducing into

25   such cells a molecule which is, or gives rise to, an anti-sense nucleic acid directed toward at least a portion of the RNA transcript of the target nucleic acid, under conditions permitting the RNAi factor to increase the degree to which the anti-sense nucleic acid inhibits expression of the target nucleic acid, thereby treating the subject.

According to a fourth aspect there is provided a method for inhibiting in a

30   subject the onset of a disorder whose alleviation is mediated by inhibiting the expression of a target nucleic acid, which method comprises the steps of

(i)   elevating the level of an RNAi factor in the subject's cells where the target nucleic acid would be expressed if the subject were suffering from the disorder, and

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(ii)   prior, concurrently with or subsequent to performing step (i), introducing into such cells a molecule which is, or gives rise to, an anti-sense nucleic acid directed toward at least a portion of the RNA transcript of the target nucleic acid, under conditions permitting the RNAi factor to increase the degree to which the anti-sense

5   nucleic acid would inhibit expression of the target nucleic acid were such expression to occur, thereby inhibiting in the subject the onset of the disorder.

According to a fifth aspect there is provided a method of determining whether inhibiting the expression of a particular target nucleic acid or the activity of its product may alleviate a disorder, which method comprises the steps of

10   (i)   elevating the level of an RNAi factor in a cell whose phenotype correlates with that of a cell from a subject having the disorder;

(ii)   prior, concurrently with or subsequent to performing step (i), introducing into the cell a molecule which is, or gives rise to, an anti-sense nucleic acid directed toward at least a portion of the RNA transcript of the target nucleic acid under conditions

15   permitting the RNAi factor to increase the degree to which the anti-sense nucleic acid inhibits expression of the target nucleic acid; and

(iii)   determining whether the cell's phenotype now correlates with that of a cell from a subject in whom the disorder has been alleviated or the disorder is not evident, thereby determining whether inhibiting the expression of the target nucleic acid or the

20   activity of its product may alleviate the disorder.

In a preferred embodiment the target nucleic acid is an endogenous nucleic acid or a part thereof, but it may also be an exogenous sequence or part thereof.

Preferably the level of the RNAi factor is elevated by introducing into the cell additional copies of, or agents which give rise to, the RNAi factor. It will be understood

25   therefore that up-regulating the expression of an endogenous RNAi factor will also achieve the same result and is contemplated herein as part of the invention.

Preferably the factor is selected from the group consisting of a gene, cDNA, RNA or a protein. More preferred is a factor selected from the group consisting of a transcriptional activator of the antisense nucleic acid, a component of the RNAi

30   machinery, a component of the DNA replication machinery and a component of translational machinery. Even more preferred is an RNAi factor which is an *res* sequence.

Also for preference the factor can be selected from the group consisting of ATP-dependent RNA helicase (*ded1*), transcriptional factor *thi1*, DNA replication protein sna41, ribosomal protein L7a, elongation factor EF-Tu and *res1* as herein defined.

Further preferred factors are represented by the res sequences which are obtainable from transformed cells designated herein W18, W20, W21, W23, W27, W28, W30, W32 and W47.

Preferably the *res* sequence is represented by any one of Seq ID Nos 1 to 4.

The preferred cell is a eukaryotic cell and even more preferred is a mammalian cell. In certain embodiments of the invention described herein the preferred cell is a *Schizosaccharomyces pombe* cell.

Preferably the antisense nucleic acid corresponds to a part only of the target nucleic acid.

According to a sixth aspect there is provided a pharmaceutical composition for use in performing the method of any one of the previous aspects comprising

(i)   an expressible nucleic acid encoding, or capable of increasing or decreasing the expression of, an RNAi factor;

(ii)   a nucleic acid encoding a molecule which is, or gives rise to, an anti-sense nucleic acid directed toward at least a portion of the RNA transcript of the target nucleic acid; and

(iii)   a pharmaceutically acceptable carrier,

wherein the nucleic acids of (i) and (ii) may be situated on the same or different molecules.

According to a seventh aspect there is provided a pharmaceutical composition for use in performing the method of any one of claims 2 to 17 comprising

(i)   an nucleic acid which is the target nucleic acid or a part thereof, or an expressible nucleic acid encoding a factor capable of elevating the intracellular level of the target nucleic acid;

(ii)   a nucleic acid encoding a molecule which is, or gives rise to, an anti-sense nucleic acid directed toward at least a portion of the RNA transcript of the target nucleic acid; and

(iii)   a pharmaceutically acceptable carrier,

wherein the nucleic acids of (i) and (ii) may be situated on the same or different molecules.

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According to an eighth aspect there is provided a cell having increased susceptibility to anti-sense-mediated inhibition of a target nucleic acid expression, which cell (i) expresses a target nucleic acid and (ii) comprises an elevated level of an RNAi factor, with the proviso that the cell is to have introduced thereinto a molecule which is, or gives rise to, an anti-sense nucleic acid directed toward at least a portion of the RNA transcript of the target nucleic acid under conditions permitting the RNAi factor to increase the degree to which the anti-sense nucleic acid inhibits expression of the target nucleic acid.

For preference the cell is a eukaryotic cell but more preferred is a mammalian cell. As indicated above, in certain embodiments of the invention described herein the preferred cell is a *Schizosaccharomyces pombe* cell.

According to a ninth aspect there is provided a method for inhibiting the expression of a target nucleic acid in a cell, which method comprises the steps of

(i)   augmenting the level of the target nucleic acid or a part thereof in the cell, and

(ii)   prior, concurrently with or subsequent to performing step (i), introducing into the cell a molecule which is, or gives rise to, an anti-sense nucleic acid directed toward at least a portion of the RNA transcript of said target nucleic acid, under conditions permitting an increase in the degree to which the anti-sense nucleic acid inhibits expression of said target nucleic acid.

According to a tenth aspect there is provided a method of increasing cellular susceptibility to anti-sense-mediated inhibition of a target nucleic acid expression, which method comprises augmenting the level of the target nucleic acid or a part thereof in a cell expressing the target nucleic acid, with the proviso that the cell is to have prior, concurrently or subsequently introduced thereinto a molecule which is, or gives rise to, an anti-sense nucleic acid directed toward at least a portion of the RNA transcript of the target nucleic acid under conditions permitting the increase in the degree to which the anti-sense nucleic acid inhibits expression of the target nucleic acid.

According to an eleventh aspect there is provided a method for treating a subject suffering from a disorder whose alleviation is mediated by inhibiting the expression of a target nucleic acid, which method comprises the steps of

(i)   augmenting the level of said target nucleic acid or a part thereof in the subject's cells where the target nucleic acid is expressed, and

(ii)   prior, concurrently with or subsequent to performing step (i), introducing into such cells a molecule which is, or gives rise to, an anti-sense nucleic acid directed toward at least a portion of the RNA transcript of the target nucleic acid, under conditions permitting an increase in the degree to which the anti-sense nucleic acid

5   inhibits expression of the target nucleic acid, thereby treating the subject.

According to a twelfth aspect there is provided a method for inhibiting in a subject the onset of a disorder whose alleviation is mediated by inhibiting the expression of a target nucleic acid, which method comprises the steps of

(i)   augmenting the level of the target nucleic acid or a part thereof in the subject's

10   cells where the target nucleic acid would be expressed if the subject were suffering from the disorder, and

(ii)   prior, concurrently with or subsequent to performing step (i), introducing into such cells a molecule which is, or gives rise to, an anti-sense nucleic acid directed toward at least a portion of the RNA transcript of the target nucleic acid, under

15   conditions permitting an increase in the degree to which the anti-sense nucleic acid would inhibit expression of the target nucleic acid were such expression to occur, thereby inhibiting in the subject the onset of the disorder.

According to a thirteenth aspect there is provided a method of determining whether inhibiting the expression of a particular target nucleic acid or the activity of its

20   product may alleviate a disorder, which method comprises the steps of

(i)   augmenting the level of the target nucleic acid in a cell whose phenotype correlates with that of a cell from a subject having the disorder;

(ii)   prior, concurrently with or subsequent to performing step (i), introducing into the cell a molecule which is, or gives rise to, an anti-sense nucleic acid directed toward

25   at least a portion of the RNA transcript of the target nucleic acid under conditions permitting an increase in the degree to which the anti-sense nucleic acid inhibits expression of the target nucleic acid; and

(iii)   determining whether the cell's phenotype now correlates with that of a cell from a subject in whom the disorder has been alleviated or the disorder is not evident,

30   thereby determining whether inhibiting the expression of the target nucleic acid or the activity of its product may alleviate the disorder.

In a preferred embodiment the target nucleic acid is an endogenous nucleic acid or a part thereof, but it may also be an exogenous sequence or part thereof.

Preferably the level of the target nucleic acid is augmented by introducing into the cell additional copies of, or agents which are capable of inducing intracellular over-expression of, the target nucleic acid. Over-expression can be achieved for an endogenous as well as an exogenous target nucleic acid. Preferably the nucleic acid

5   used for augmenting content of the target nucleic acid is a fragment, derivative or analogue of the target nucleic acid. However it will be understood that the entire native sequence of the target nucleic acid may be employed.

Conveniently, the target nucleic acid may be coupled to a selectable marker.

The preferred cell is a eukaryotic cell and even more preferred is a mammalian

10   cell. In certain embodiments of the invention described herein the preferred cell is a *Schizosaccharomyces pombe* cell.

Preferably the antisense nucleic acid corresponds to a part only of the target nucleic acid.

According to a fourteenth aspect there is provided a cell having increased

15   susceptibility to anti-sense-mediated inhibition of a target nucleic acid expression, which cell (i) expresses said target nucleic acid and (ii) comprises an elevated level of said target nucleic acid, with the proviso that the cell is to have introduced thereinto a molecule which is, or gives rise to, an anti-sense nucleic acid directed toward at least a portion of the RNA transcript of the target nucleic acid under conditions permitting the

20   RNAi factor to increase the degree to which the anti-sense nucleic acid inhibits expression of the target nucleic acid.

The preferred cell is a eukaryotic cell and even more preferred is a   ·
Schizosaccharomyces pombe cell.

According to a fifteenth aspect there is provided a method of identifying a

25   cellular factor capable of effecting and/or modulating expression of a target nucleic acid in a cell having the target nucleic acid and a nucleic acid which is an antisense of the target nucleic acid or part thereof, which method comprises over-expressing said factor in the cell and wherein the expression of the target nucleic acid is capable of being enhanced or only partially suppressed.

30   According to a sixteenth aspect there is provided a factor identified by the method of the fifteenth aspect.

The preferred factors can be selected from the group consisting of a gene, cDNA, RNA or a protein. More preferred are factors selected from the group consisting of a

transcriptional activator or the antisense nucleic acid, a component of the RNAi
machinery, a component of the DNA replication machinery and a component of
translational machinery. Even more preferred is a factor having an *res* sequence.

Preferred factors can also be selected from the group consisting of ATP-
dependent RNA helicase (*ded1*), transcriptional factor *thi1*, DNA replication protein
sna41, ribosomal protein L7a, elongation factor EF-Tu and *res1* as herein defined.

According to a seventeenth aspect there is provided an RNAi factor which is an
res sequence obtainable from transformed cells designated herein W18, W20, W21,
W23, W27, W28, W30, W32 and W47.

According to an eighteenth aspect there is provided an RNAi factor which is an
res sequence represented by Seq ID Nos 1 to 4.

According to a nineteenth aspect there is provided a *Schizosaccharomyces
pombe* cell having a target nucleic acid or a part thereof and a antisense nucleic acid or a
part thereof which corresponds to the target nucleic acid or a part thereof, wherein the
expression of the target nucleic acid is capable of being enhanced or only partially
suppressed.

The term "inhibiting expression of a target nucleic acid" as used in the context of
the present invention is intended to encompass, but not be limited to, reduction or
elimination of gene expression, whether or not the target nucleic acid is a gene, or a part
thereof, introduced into the cell or it is an endogenous gene.

The term "RNAi factor" is intended to include in its scope any naturally
occurring, modified or synthetic molecule capable of enhancing RNAi activity. This
definition includes in its scope the factors referred to herein as *RNAi enhancing
sequences (res)*. The term *res* may be used herein interchangeably with the term *aes*
(*anti-sense enhancing sequences*).

It will be understood by those skilled in the art that "elevating RNAi factor
level" can be achieved by transfection, upregulation or other means known in the art.

The term "anti-sense nucleic acid molecule" as used in the context of the present
invention is intended to encompass RNA or DNA and contain region(s) related to a
specific target RNA transcript or its gene. It is also intended to include molecules
giving rise to antisense sequences, including inverted repeat, sense RNA, etc.

The term "conditions permitting the RNAi factor to increase the degree to
which the anti-sense nucleic acid molecule inhibits expression of the gene"

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is intended to include the concept of inhibition of gene expression which is high enough

to be effective, but not so high as to harm the cell. The effective concentration range

can be determined using routine methodology known in the art.

Reference to a "subject" is intended to include human, animal (mouse), plant, or

5   other life forms.

Reference to a "cell" is intended to encompass eukaryotic cells such as yeast,

mammalian, plant, etc

The term "disorder" will be understood to inlcude viral infection (HIV, HPV,

etc), cancer, autoimmune disease and other chronic and acute diseases.

10   The term "phenotype" as used in the context of the present invention is intended

to include cell staining, morphology, growth, and similar characteristics.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Target genes and antisense fragments. **a,** The strain KC4-6 expresses low

levels of *lacZ* RNA. The *lacZ* expression cassette is integrated at the *ura4* locus on

15   chromosome III and is composed of the SV40 early promoter, the *E. coli lacZ* gene and

the SV40 3' processing signal. The 5' and 3' flanking DNA sequences of the *ura4* locus

are as indicated. **b,** The strain RB3-2, which expresses high levels of *lacZ* RNA,

contains an expression cassette containing the *S. pombe adh1* promoter, the *lacZ* coding

region, and the *S. pombe ura4 3'* processing signal. **c,** The full-length 3.5 kb antisense

20   *lacZ* fragment is shown. The crippled sense fragment was generated by end-filling the

*Cla*I cut *lacZ* vector and religating on itself. **d,** The AML1 strain contains a *c-myc-lacZ*

fusion cassette integrated at the *ura4* locus. Exons 2 and 3 of the human *c-myc* gene

were employed as a target. The relative position of the *c-myc* antisense fragment is

shown. Transcription initiation sites are indicated by the bent arrows. The straight

25   arrows represent the normal direction of transcription for a particular DNA fragment.

Figure 2. dsRNA-mediated gene silencing in fission yeast. **a,** A fission yeast strain

containing the integrated *lacZ* gene under control of the strong *adh1* promoter was

transformed with *lacZ* antisense vectors. The antisense gene was under control of the

weak *nmt41* promoter (low antisense), the strong *nmt1* promoter in single copy (15)

30   (medium antisense), or the *nmt1* promoter in multi-copy (high antisense). For the strain

expressing maximum antisense RNA two episomal *nmt1*-driven antisense vectors

containing different selectable markers were co-transformed into the target strain. **b,** A

fission yeast strain containing the integrated *lacZ* gene under control of the weak SV40

promoter (low target) or strong *adh1* promoter (high target) was transformed with the

antisense *lacZ* vector or antisense *lacZ* and sense *lacZ* vectors. Strains were co-

transformed with vectors containing appropriate selectable markers to complement

5   auxotrophy. **c,** The *lacZ* inverted repeat vector was expressed in the high-expressing

lacZ target strain. The strain expressing the antisense construct only is also indicated. **d,**

A fission yeast strain containing an integrated *c-myc-lacZ* fusion gene was transformed

with the sense construct, the antisense construct, or both. Antisense and sense constructs

were also expressed in the high-expressing *lacZ* strain. All strains were transformed

10   with appropriate control plasmids to complement auxotrophy.

Figure 3. Effect of co-expressing single copies of sense and antisense genes. Strains

containing target *lacZ* alone (RB3-2), the target and a single copy of the antisense gene

(K40-7), and the target and both the antisense and frameshifted *lacZ* genes (M62-1)

were assayed for β-galactosidase activity. pREP2 and pREP4 are parental plasmids

15   containing the *LEU2* and *ura4* selectable markers respectively. Strains were transformed

with the appropriate control plasmids to complement auxotrophy. For each sample three

independent colonies were assayed in triplicate.

Figure 4. RNA expression profiles in fission yeast strains. **a,** The target strain RB3-2

was transformed with the *lacZ* inverted repeat vector (pM53-1) or co-transformed with

20   antisense (pGT2) and sense (pM54-3) vectors and grown to mid-log in the absence of

thiamine. Total RNA from each strain was separated on a 1% denaturing agarose gel,

transferred to a nylon membrane and probed with the radioactively labeled 2.2 kb *nmt1*

fragment. The episomal *lacZ* signal was normalized with the endogenous *nmt1*

transcript and quantitated by phosphorimage analysis. The relative level of episomal

25   *lacZ* expression is shown. **b,** The strain M38-1 which contains a single copy stably

integrated *lacZ* inverted repeat gene was grown to mid-log in the absence of thiamine,

its RNA isolated and analyzed by Northern hybridization. The relative steady-state level

of the "panhandle" *lacZ* RNA is indicated in comparison to episomally expressed

antisense *lacZ* RNA.

30   **Figure 5.** In vivo dsRNA assay. **a,** The constructs employed for assaying the ability of

the *lacZ* inverted repeat to form an intramolecular RNA duplex are shown. Each vector

contains a functional *lacZ* fragment which gives the transformed strain a blue color

phenotype when grown in the presence of X-GAL. The predicted secondary structure of
the pM81-2 generated transcript is indicated. **b,** The strain 2037 was transformed with
the vectors pM85-1, pM91-1 and pM81-2 in the absence of thiamine. Single colonies
were streaked on minimal media plates and overlayed with 0.5% agarose medium

5   containing 500 µg/ml X-gal and 0.01% SDS.

Figure 6. The effect of *ded1* on dsRNA-mediated gene regulation. **a,** The *ded1* gene
was co-expressed in a fission yeast strain containing the integrated *lacZ* gene. β-
galactosidase activity of the strain transformed with and without the antisense *lacZ*
vector is shown. **b,** The target strain was transformed with a vector expressing a short

10   *lacZ* antisense gene or with both the short *lacZ* antisense vector and the *ded1*-expressing
vector. Strains were transformed with appropriate control plasmids to complement
auxotrophy.

Figure 7. Over-expression of *thi1* in an antisense *lacZ* expressing strain.
β–galactosidase activity of the target strain RB3-2 transformed with and without the

15   antisense *lacZ* vector is shown. Over-expression of the *thi1* gene in the antisense
expressing strain is also indicated. Strains were transformed with appropriate control
plasmids to complement auxotrophy.

Figure 8. Over-expression screening strategy for RNAi modulating factors. A target
strain containing the integrated *lacZ* gene under control of the *adh1* promoter and the

20   episomal vector containing the *nmt1*-driven *lacZ* antisense gene is transformed with an
S. pombe cDNA library. Library fragments are driven by the *nmt1* promoter.
Transformants are individually screened for a change in the *lacZ*-encoded blue colony
colour phenotype. These transformants are then quantitatively assayed for
β–galactosidase activity in the presence and absence of thiamine. The antisense vector

25   is then segregated out of transformants showing a cDNA dependent modification of
lacZ suppression. A tertiary β–galactosidase assay is performed to determine if the
effect is dependent on the presence of dsRNA. cDNA vectors are recovered from strains
of interest, sequenced, and subjected to BLASTN analysis.

Figure 9. Over-expression screen of a *S. pombe* cDNA library. **a,** Transformants were

30   grown on minimal media plates and over-layed with X-GAL-containing medium. Those
which showed a reduced blue colour-phenotype (black arrow) were analysed further.
Transformants demonstrating an enhanced blue-colour phenotype were also identified

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(white arrow). b, Transformants which showed a visual reduction in the blue phenotype were assayed for β–galactosidase activity in liquid culture in the absence of thiamine. Thiamine was added to the medium to demonstrate that the enhanced *lacZ* suppression was dependent on RNA expression. Transformants were again assayed for

5 β–galactosidase activity following antisense vector segregation. c, Over-expression of unique *res* genes in dsRNA-expressing strains.

Figure 10. lacZ panhandle-mediated gene silencing. (A) The lacZ panhandle construct contains the full-length crippled lacZ gene which is followed by the inverted 5' 2.5 kb   .
lacZ fragment. Intramolecular hybridization generates an RNA with 2.5 kb RNA duplex

10 and a 1 kb loop. (B) The relative steady-state level of the episomally expressed panhandle lacZ RNA (7.0 kb) is shown in comparison to episomally expressed antisense lacZ RNA (4.5 kb). The lacZ signals were normalized to the endogenous nmt1 transcript (1.3 kb) and quantitated by phosphorimage analysis. (C) The target strain was transformed with the episomally expressed lacZ panhandle and analyzed for b-

15 galactosidase activity. The appropriate plasmids were co-introduced to complement auxotrophy. At least three independent colonies were assayed in triplicate for each strain. Transformants were assayed in the presence of thiamine to abrogate expression of the panhandle RNA (hatched). (D) The target lacZ strain was transformed with the panhandle vector only or both the panhandle and aes2 vector.

20 **Figure 11.** Co-expression of ded1 and lacZ antisense genes. b-galactosidase activity of the target strain co-transformed with the ded1 gene and the antisense lacZ vectors is shown. Ded1 was expressed from the ura4-based plasmid pREP4 while the antisense genes were expressed from the LEU2-based plasmid pREP2. Strains expressing antisense RNAs or ded1 RNA alone are also indicated. Strains were transformed with

25 appropriate control plasmids to complement auxotrophy. Three independent colonies were assayed in triplicate for each strain.

Figure 12. DNA sequence for *aes1* factor

Figure 13. DNA sequence for *aes2* factor

Figure 14. DNA sequence for *aes3* factor

Figure 15. DNA sequence for *aes4* factor

5   **DESCRIPTION OF THE PREFERRED EMBODIMENTS**

Using the *lacZ* fission yeast model to investigate features of antisense-RNA technology in vivo (1-4), it has been shown that the degree of target gene suppression is dependent on the level of antisense RNA (Fig. 2A)(1). During the course of developing this model it was suprisingly found that by increasing the steady-state levels of the *lacZ*

10   target mRNA 20-fold in the presence of the same pool of antisense RNA that antisense-mediated suppression was not only maintained, but enhanced (Fig. 2B). To verify that this effect was independent of the target mRNA a *lacZ* gene containing a frameshift mutation (leading to an inability to translate the β-galactosidase enzyme) was co-expressed in antisense-expressing strains. Again, additional stimulation of *lacZ*

15   inhibition was observed (Fig. 2B). These observations are consistent with the potential formation of additional dsRNA and consequent stimulation of gene silencing. They also indicate that the dsRNA-mediated gene silencing is dose-dependent in this system. To further show that dsRNA was the key component in target gene inhibition in fission yeast dsRNA was generated by expressing a *lacZ* inverted repeat with 2.5 kb of internal

20   complementarity in the presence of *lacZ* target mRNA. Expression of this construct also reduced β-galactosidase activity in a *lacZ* expressing strain to a similar level to the full-length antisense RNA (Fig. 2C). Overall, these data suggested that increasing the potential formation of dsRNA, but not necessarily an antisense RNA:target mRNA hybrid, resulted in efficient interference of target gene expression in *S. pombe*.

25   To test the ability of dsRNA to specifically interfere with another target sequence in fission yeast, sense and antisense *c-myc* sequences were co-expressed in a strain containing an integrated *c-myc-lacZ* fusion cassette (3). A 792bp antisense *c-myc* fragment from exon 2 of the human *c-myc* gene was previously found to suppress β–galactosidase activity within the *c-myc-lacZ* fusion target strain by 47% (3).

30   β–galactosidase assays demonstrated that co-expression of the antisense and sense *c-myc* constructs in the target strain enhanced *c-myc* suppression compared with the antisense *c-myc* vector alone (Fig. 2D). Transformation of the strain expressing only the

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lacZ target with the antisense and sense *c-myc* constructs resulted in no down-regulation of β–galactosidase activity indicating that the action of dsRNA is sequence-specific (Fig. 2D). All of the above observations, as well as the reduction in target mRNA levels in response to additional dsRNA (data not shown), are consistent with features

5   associated with RNAi. This is the first example of dsRNA-mediated gene regulation in yeast and, unlike RNAi in all other organisms except zebrafish, appears to be dependent on the concentration of intracellular dsRNA. The variation in RNAi efficacy between organisms may be due to the absence or under-expression of some components of the RNAi pathway or the presence of RNAi inhibitors. We have now further developed this

10   genetic model for identification of factors involved in RNAi.

Without wishing to be bound by any particular mechanism, it is probable that a multi-protein complex exists which mediates the post-transcriptional degradation of target mRNA in RNAi. Several genes which are involved in the RNAi phenomenon have been identified through genetic screens in *Neurospora* and nematodes. These genes

15   include an RNA-dependent RNA polymerase (*qde-1, ego-1*) a RecQ DNA helicase (*qde-3*), an RNase D homologue (*mut-7*), and a putative translation initiation factor (*rde-1, qde-2*). The genes *mut-2, rde-2, rde-4*, and *rde-7* have also been identified as being involved in either the initiation or maintenance of RNAi. Additionally, *Drosophila* cell free assays have shown that RNAi is mediated by nuclease degradation of the targeted

20   mRNA while a 21-25 nt RNA species appears to be integral to specific post-transcriptional genetic interference. RNAi has also been shown to be dependent on ATP which may be required for strand dissociation of dsRNA. However, a missing component of the proposed multi-protein complex in the RNAi pathway is the implied RNA helicase.

25   The dose-dependency of dsRNA-mediated gene silencing in fission yeast described above has allowed us to use an over-expression strategy to identify genes involved in RNAi. In comparison to mutagenesis strategies, over-expression can enable the identification of genes which are otherwise essential for cell viability. Also, cellular factors that quantitatively enhance or reduce RNAi activity can be determined. The first

30   gene that was tested in mediating RNAi activity in the present model was the *nmt1* transcription factor *thi1*. This gene has been shown to specifically up regulate *nmt1* expression when overexpressed in fission yeast. As we have previously shown that

antisense RNA-mediated gene suppression is dose dependent in *S. pombe* (1) it was
hypothesized that over-expression of *thi1* would result in increased production of *nmt1*-
driven antisense *lacZ* RNA and a consequent enhancement in target gene suppression.
The *thi1* open reading frame was PCR amplified and subcloned into pREP4 as a *Bam*HI

5   fragment. This vector was then transformed into RB3-2/pGT2 and β-galactosidase
assays performed. As predicted, *lacZ* suppression was enhanced when compared to a
strain expressing antisense RNA alone (Fig. 7). This result indicated that host-encoded
factors are present which affect the robustness of dsRNA-mediated gene suppression in
fission yeast and also validated the overexpression strategy.

10   The second gene investigated has been the *S. pombe* ATP-dependent RNA
helicase gene, *ded1*. *Ded1* is an essential gene which has previously been characterized
as a suppressor of sterility, a suppressor of checkpoint and stress response, and a general
translation initiation factor. According to current models of dsRNA-mediated gene
regulation an ATP-dependent RNA helicase may be required in conjunction with a

15   dsRNA-dependent RNA polymerase for the formation of short single-stranded RNA
fragments which specifically degrades target mRNA. It was therefore reasoned that
over-expression of this gene in fission yeast could enhance the efficiency of dsRNA-
mediated gene silencing by stimulating the unwinding of dsRNA. Co-expression of the
ded1 vector with the antisense *lacZ* vector significantly enhanced dsRNA-mediated *lacZ*

20   inhibition by a further 50% compared to the antisense expressing strain (Fig. 6A). When
ded1 was over-expressed in the absence of the antisense *lacZ* vector, β-galactosidase
activity was comparable to control strains indicating that its effect was dependent on the
presence of dsRNA (Fig. 6A). The *ded1* vector was also co-expressed with a short
antisense *lacZ* vector which has previously been shown to be less effective than the full-

25   length antisense gene (16). Again, the over-expression of *ded1* stimulated dsRNA-
mediated *lacZ* inhibition (Fig. 6B). It was thus shown that over-expression of an ATP-
dependent RNA helicase greatly enhances RNAi in fission yeast.

           By over-expressing a cDNA library in a dsRNA fission yeast model we have
identified four novel genes with potential roles in the RNAi pathway. All of the known

30   genes identified are essential and have natural associations with DNA, RNA or nucleic
acid binding proteins, consistent with the expectation of RNAi cellular factors. In
addition, two of the three known cDNAs encoded proteins involved in the process of

translation. These findings along with our previous identification of an ATP-dependent RNA helicase (Raponi & Arndt, submitted) and reports of the involvement of a translation initiation factor in RNAi and the co-purification of ribonuclease activity with ribosomal fractions, suggests that this may be one site at which RNAi functions.

5   Alternatively, the identification of such varied proteins as DNA helicases and translational components as part of the RNAi machinery implies that particular cellular proteins may be recruited into more than one multiprotein complex. Under the latter conditions, over-expression of these specific proteins may result in the generation of an RNAi complex that recognises dsRNA and mediates target gene suppression. Certain of

10   these proteins may be rate-limiting or rate-determining in RNAi and only through supplementation of these factors are their roles in RNAi uncovered. It is proposed, without wishing to be bound by any particular mechanism, that in addition to the previously identified core proteins in the RNAi multiprotein complex(es), additional factors such as EF Tu, L7a, sna41 and res1 may be enlisted for a role in dsRNA-

15   mediated gene suppression.

The over-expression strategy described overcomes some limitations associated with mutagenesis by identifying essential genes with a role in RNAi. In addition, this strategy complements these other systems by allowing the isolation of cellular factors that modify the efficacy of RNAi in vivo. The roles of these modulators of RNAi may

20   be varied and include recognition and amplification of the dsRNA, delivery of the small 21-25 nt dsRNAs to the target mRNA, association between the antisense and target mRNA strands, and RNAi complex formation. Alternatively, these modulators may control the rate of RNAi or the formation of different complexes within cell types or for different forms of post-transcriptional gene silencing. The fact that over-expression of

25   specific RNAi modulators enhanced dsRNA-mediated gene regulation in fission yeast indicates that a similar approach could be used to identify RNAi modulators in other organisms. In addition, the co-expression of these factors with different forms of post-transcriptional gene silencing including co-suppression, quelling, and antisense RNA could be one way of enhancing the efficacy of these methods. This may be especially

30   important for application of RNAi to mammalian cells and tissues or to genes which have been somewhat recalcitrant to this form of regulation.

The present invention demonstrates for the first time the intrinsic involvement of an ATP-dependent RNA helicase as a key component in RNAi. Further, it can be rate

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limiting, as the over-expression leads to increased RNAi activity in this system. This ability of the *ded1*-encoded RNA helicase is consistent with its activities as a member of the DEAD box family of helicases, with their three core domains of ATPase, RNA helicase, and RNA binding activities. These could allow the enzyme to enhance gene

5   suppression as follows: (i) in a dissociative mechanism it could mediate either the unwinding of dsRNA to generate a cRNA in conjunction with an RNA-dependent RNA polymerase or strand separation of fragmented dsRNA to enhance binding to homologous transcripts, and/or (ii) in an associative mechanism it could catalyse the ATP-dependent exchange of the sense strand of the short dsRNA with the target mRNA.

10   The presence of *ded1* homologues in other organisms displaying RNAi further supports the general involvement of this component in the RNAi machinery.

The present invention also provides for the first time a novel and quantitative genetic system based on *S.pombe*, for rapidly identifying essential cellular factors involved in RNAi. The use of this model has enabled verification of RNA helicase

15   activity as a critical contributor to efficient RNAi activity and isolated novel RNAi factors including EF Tu, L7a, *sna41*, and an unidentified gene *res1*. The present invention also demonstrates that gene silencing may be enhanced by concomitant expression of such RNAi factors.

The invention will now be described more particularly with reference to non-

20   limiting examples of certain preferred embodiments of the invention.

EXAMPLE 1: Materials and methods used to exemplify the invention

S. pombe media and manipulations.

All yeast strains were maintained on standard YES or EMM media (6). Repression of *nmt1* transcription was achieved by the addition of thiamine to EMM

25   media at a final concentration of 4 µM (7). Yeast cells were transformed with plasmid DNA by electroporation (8) and stable integrants were identified as previously described (6). A glass bead procedure (9) was used to isolate genomic DNA which was used for Southern analysis and PCR diagnosis. Total RNA was extracted as previously described (10).

30   *S. pombe* strain and plasmid construction.

Construction of the low expressing *lacZ* strain, KC4-6 (*h⁻, ura4::SV40-lacZ, leu1-32)*, has been previously described (2). The strain RB3-2 (*h⁻, ura4::adh1-lacZ,*

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leu1-32) which expresses higher levels of β–galactosidase has also been previously described (1). The target strain which contains the *c-myc:lacZ* fusion has previously been described (3).

The construction of the long *lacZ* antisense containing episomal plasmid, pGT2,
5   and corresponding control plasmids have been described (2). Plasmid pREP4-As was generated by subcloning the *lacZ BamHI* fragment contained in pGT2 into the plasmid pREP4 (11). pREP4 is identical to pREP1 except that the *S. cerevisiae LEU2* gene has been replaced with the *S. pombe ura4* selectable marker. To decrease the steady-state level of episomally expressed antisense *lacZ*, plasmids pREP42-As and pREP82-As
10   were constructed by subcloning the *lacZ BamHI* fragment from pGT2 into pREP42 and pREP82, respectively. These plasmids are derivatives of pREP4 with mutations in the TATA box of the *nmt1* promoter (12). The crippled *lacZ* vector, pGT62, was generated by end-filling the *ClaI* site of pGT2 and re-ligating (2). This frameshifted fragment was then subcloned into the *BamHI* site of pREP4 to generate the plasmid pM54-3.
15   The *lacZ* panhandle integration vector, pM30-8, was generated by first introducing a *NotI* site into the *XmaI* site of pRIP1/s (11) using the self-complementary linker 5' CCG GGC GGC CGC 3' to generate pL121-14. The 2.5 kb sequence of the 5' end of the frameshifted *lacZ* gene (2) was then PCR-amplified to give it *NotI* ends using the forward primer 5' ATGCGGCCGCAATTCCCGGGGATCGAAAGA 3' and
20   reverse primer 5' ATGCGGCCGCAATGGGGTCGCTTCACTTA 3'. This product was cloned using a TA cloning kit (TOPO: Invitrogen, San Diego, CA, USA) and then subcloned into the *NotI* site of pL121-14 in the antisense orientation. The integrating vector was introduced into target strains in single copy by using the *sup3-5/ade6-704* complementation system (13). The full-length frameshifted *lacZ* fragment was then
25   introduced into the *BamHI* site of this vector in the sense orientation upstream of the 2.5 kb antisense fragment to generate pM30-8. The episomal version of this vector (pM53-1) was made by removing the *PstI sup3-5* fragment and introducing the autonomous replicating sequence as an *EcoRI* fragment (11). For testing the ability of the panhandle construct to form dsRNA in vivo, the frameshifted *lacZ* fragment was replaced with the
30   functional *lacZ* fragment in the vectors pM54-3 and pM53-1, to generate the vectors pM85-1 and pM81-2, respectively. The vector pM91-1, which is unable to form a *lacZ*

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panhandle transcript, was generated by removing the 2.5 kb *NotI lacZ* fragment from pM81-2 and reintroducing it in the sense orientation.

Generation of the *c-myc* antisense construct, pCM-17, has been described elsewhere (3). The 792 bp *BglII c-myc* fragment from pCM-17 was subcloned into the

5   *Bam*HI site of pREP4 in the sense orientation to generate pN12-1.

The *ded1,* and *thi1,* open reading frames were amplified from fission yeast genomic DNA (strain 1913). *ded1* was amplified to give it *Bam*HI ends using the forward primer 5' ATGGGATCCCAACCAAACACTTCAACTCAG 3' and the reverse primer 5' ATGGGATCCTCAGAAGCCTGTGCATAACAC 3'. *thi1* was amplified to

10   give it *Bgl*II ends using the forward primer 5' ATGAGATCTGTGGTTGGTATTCTAGAGAGA 3' and the reverse primer 5' ATGAGATCTAACAAAGACCTGCAAAAAACC 3'. PCR products were purified (Qiagen PCR purification kit), digested with either *Bam*HI or *Bgl*II, gel-purified (Qiagen), and subcloned into the *Bam*HI site of pREP4 in the sense orientation.

15   Southern and Northern analysis.

Nucleic acid electrophoresis and membrane transfer was performed as described (14). Southern and Northern blots were hybridized using ExpressHyb solution according to the manufacturer's instructions (Clontech Laboratories). DNA probes were ^{32}P-labelled using the Megaprime labelling kit (Amersham). Probes included a 960 bp

20   *Bam*HI/*Cla*I *lacZ* fragment from pI2-1 (1), a 570 bp *Hind*III/*Eco*RI *ura4*-3' fragment from pGT113 (15), and a 2.2 kb *Pst*I/*Sac*I *nmt1* fragment from pRIP1/s. Radioactive signals were detected by autoradiography and quantitated by phosphorimage analysis (ImageQuant; Molecular Dynamics).

Plasmid distribution analysis.

25   Plasmid co-transformants of strain RB3-2 were grown under selective conditions to a cell density of $1-2 \times 10^7$ cells/ml. A serial dilution of each culture was performed and cells plated for single colonies in triplicate onto each of YES, EMM, EMM+leucine, and EMM+uracil agar media. The number of colonies grown on YES was taken as the total number of viable cells, while colonies growing on EMM

30   represented the cells in the sampled population that contained both the *ura4*-containing (pREP4-based) and the *LEU2*-containing (pREP1-based) plasmids. Cells containing either the *ura4*-containing plasmid or the *LEU2*-containing plasmid were identified

from the EMM+leucine and EMM+uracil plates respectively. The ratio of the number of colonies grown on selective media to the total number of viable colonies was used as the quantitative measure of the proportion of cells in the population, grown under selection, which contained plasmids.

5   β-galactosidase assays.

The expression of the *lacZ* gene-encoded product, β–galactosidase, was quantitated using a cell permeabilization protocol as previously described (Raponi et al., 2000). A semi-quantitative overlay assay was also employed for rapid screening of yeast transformants (3).

10   Isolation of *S. pombe* cDNA clones that alter antisense RNA efficacy.

The *S. pombe* cDNA library was originally constructed in pREP3Xho by Bruce Edgar and Chris Norbury (5). The vector pREP3Xho is derived from pREP3 which contains the *LEU2* marker and inserts are under control of the *nmt1* promoter (11). A total of 5 μg of library DNA was transformed into the strain RB3-2 containing the

15   episomal antisense *lacZ* vector pREP4-lacZAS and grown in EMM liquid media to the mid-logarithmic phase. Transformants were then plated on EMM solid media and grown at 30°C for 3 days. Colonies were over-layed with medium containing 0.5 M sodium phosphate, 0.5% agarose, 2% dimethylformamide, 0.01 % SDS, and 500 μg/ml X-GAL (Progen, Australia). Plates were then incubated at 37°C for 3 hrs, colonies of

20   interest recovered and assayed for β–galactosidase activity.

Plasmid segregation.

Strains were plated on EMM containing limiting uracil and 1 mg/ml 5-fluoroorotic acid (6). Strains were then replica plated on both selective and non-selective media. Those colonies that did not grow on selective media were identified as having lost the *ura4-*

25   containing antisense plasmid. To determine the mitotic stability of pREP-based plasmids we used the plasmid segreagtion method previously described (16). This method indicated that up to 30% of the cells grown in selective media did not contain the resident plasmid.

EXAMPLE 2: Effect of steady-state level of target mRNA and antisense RNA on

30   **antisense efficacy.**

To determine the role of target mRNA steady-state levels in antisense-mediated gene suppression, we investigated the ability of a long *lacZ* antisense RNA (2) to

regulate *lacZ* target genes under control of both weak and strong constitutive promoters. The low-level expressing strain, KC4-6, contained the *lacZ* gene driven by the SV40 early promoter integrated at the *ura4* locus in chromosome III (Fig. 1A; (2)). The high-level *lacZ* expressing strain, RB3-2, was constructed by placing the *lacZ* gene under

5   control of the strong fission yeast *adh1* promoter and the 3' processing signal from the *ura4* gene and integrating this expression cassette at the *ura4* locus (Fig. 1B; (1)). Characterization of both strains indicated that RB3-2 expressed 20-fold more *lacZ* mRNA than KC4-6 while an approximate 40-fold increase in β–galactosidase was also detected (data not shown). Both strains were transformed with the *lacZ* antisense gene-

10   containing plasmid (pGT2) and its corresponding sense control plasmid (pGT62). β–galactosidase assays indicated that the antisense RNA suppressed β–galactosidase activity by 45% in KC4-6 while the same RNA reduced β–galactosidase activity by 52% in RB3-2 (Fig. 2B). No reduction in β–galactosidase activity was seen in either strain when transformed with the control plasmid pGT62 which expresses a crippled

15   version of *lacZ*. These results indicated that despite a 20-fold increase in the steady state level of the *lacZ* target mRNA in RB3-2 the efficacy of antisense-mediated down regulation was not only maintained, but enhanced. The increase in gene suppression demonstrated with strain RB3-2 was also seen when antisense molecules targeted to subregions of the *lacZ* target gene were used, but was not as pronounced. These data

20   suggested an increase in target mRNA can result in enhanced target gene suppression.

It has previously been demonstrated that a stably integrated antisense *lacZ* gene acts in a dose dependent fashion with the steady-state levels of antisense RNA being dependent on genomic position effects and transgene copy number (1). Here the role of the steady-state level of episomally expressed antisense RNA in both the low *lacZ*

25   expressing target strain, KC4-6, and the high *lacZ* expressing target strain, RB3-2, was investigated. The expression of *lacZ* antisense RNA was increased by co-transforming KC4-6 and RB3-2 with plasmids pGT2 and pREP4-As, each of which contains the *lacZ* antisense gene under control of the *nmt1* promoter, but different selectable markers. To decrease the steady-state level of antisense RNA, the plasmids pREP42-As and

30   pREP82-AS were employed. The *nmt1* promoter in these vectors contain deletions in the TATA box sequence which affect the level of transcription, but have no impact on the site of transcription initiation or thiamine repressibility (12). Each of the different

antisense gene-containing plasmids was co-transformed with a control plasmid to complement auxotrophy where appropriate. This resulted in a set of co-transformants of both RB3-2 and KC4-6 each containing the same *lacZ* antisense gene, but with different promoter capacities. Each co-transformant was analyzed for antisense RNA steady-state levels and β–galactosidase activity. Table 1 indicates that with both strains the degree of target suppression is enhanced with the increase of antisense *lacZ* RNA expression.

Table 1. Effects of varying the level of antisense RNA in strains expressing both low and high level target.

Strain	Plasmids	Descriptions	β-gal. activity (units)	% suppression[a]	Relative antisense RNA level[b]
KC4-6	pREP2, pREP4	Vector	1.07	0	0
	pREP2, pREP82-AS	Low As	1.10	0	0.04
	pREP2, pREP42-AS	Medium As	1.07	0.1	0.14
	pREP2, pREP4-AS	Full As-*ura4*	0.75	30	1.0
	pGT2, pREP4	Full As-*LEU2*	0.70	34.6	2.2
	pGT2, pREP4-AS	Double-As	0.54	49.2	5.6
RB3-2	pREP2, pREP4	Vector	48.5	0	0
	pREP2, pREP82-AS	Low As	44.0	9.4	0.05
	pREP2, pREP42-AS	Medium As	43.9	9.6	0.18
	pREP2, pREP4-AS	Full As-*ura4*	27.2	43.9	1.0
	pGT2, pREP4	Full As-*LEU2*	24.9	48.7	1.9
	pGT2, pREP4-AS	Double-As	16.6	65.8	5.4

a. The % suppression of β-galactosidase activity was determined by expressing each β-galactosidase activity as a percentage of the activity found in the pREP2,pREP4 co-transformant.

b. Within each strain the steady-state level of long *lacZ* antisense RNA was normalized to *nmt1* mRNA and then expressed relative to the level observed in the pREP2, pREP4-As co-transformant.

These data agree with the previous report that antisense RNA-mediated gene inhibition is dose dependent in *S. pombe* (1). However, for each co-transformant the

degree of β-galactosidase suppression was greater by 10-15% in the high *lacZ*
expressing strain, RB3-2, than the low *lacZ* expressing strain, KC4-6, again
demonstrating that low levels of target mRNA can limit antisense efficacy.

EXAMPLE 3: Effect of increasing sense RNA on antisense RNA-mediated target

5   **gene suppression.**

        To determine whether the increase in gene suppression was due to formation of
an antisense RNA:target mRNA hybrid or an antisense RNA:sense RNA hybrid, a
version of *lacZ* which is unable to be translated into functional β-galactosidase was co-
expressed in strains expressing the *lacZ* antisense gene. If antisense RNA was required

10   to hybridize to target mRNA for inhibition of the gene expression pathway, then over-
expression of the sense RNA would compete with the target for the available antisense
molecules and a decrease in *lacZ* gene suppression would result. Initially, both antisense
and sense *lacZ* vectors were integrated in single copy into separate target strains and
then crossed with each other. In strains containing the antisense gene alone,

15   β-galactosidase activity was reduced by approximately 40% (Fig. 3; (1)) while there
was no reduction in the strain expressing sense *lacZ* alone (data not shown). However,
in the strain expressing both complementary transcripts (M62-1), β-galactosidase
activity was reduced by 50% (Fig. 3). These results suggested that increasing the
intracellular concentration of sense RNA in the presence of antisense RNA, and

20   therefore potentially generating more dsRNA, stimulates target gene suppression.

        To further increase the potential formation of intracellular dsRNA an episomal
sense *lacZ* plasmid (pM54-3; Fig. 2B) was co-transformed with the episomal antisense
lacZ plasmid, pGT2, into the target strain RB3-2. Both of these sequences were
expressed by the strong conditional *nmt1* promoter and RNA analysis showed that each

25   transcript was being expressed at high levels when grown in the absence of thiamine
(Fig. 4A). When both RNAs were co-expressed in RB3-2, the target *lacZ* was
suppressed by 65% compared to 50% in the strain expressing the antisense plasmid
alone (Fig. 2B). Northern analysis demonstrated that the total relative level of
episomally expressed *nmt1*-driven transgenes was approximately 60% higher than when

30   either vector was expressed alone (Fig. 4A). Although expression of these
complementary RNA transcripts is at a high level compared with single copy integrants
only an additional 15% suppression was observed.

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One explanation for the absence of higher levels of target gene suppression is plasmid segregation. *S. pombe* undergoes asymmetric segregation, with the result that mitosis produces a daughter cell which lacks the segregating plasmid (17). Therefore, when the 65% suppression level seen in the total population is corrected for only those

5   cells containing the *ura4*-based and *LEU2*-based plasmids the level of suppression approaches approximately 100% (data not shown). Overall these data suggest that increasing the potential formation of dsRNA, but not necessarily an antisense RNA:target mRNA hybrid, is required for efficient interference of target gene expression in *S. pombe*. However, unlike the phenomenon of RNAi seen in plants and

10   nematodes, the dsRNA-mediated gene suppression demonstrated in fission yeast seems to be dependent on the concentration of intracellular dsRNA or a threshold level of dsRNA is required to invoke potent gene silencing.

EXAMPLE 4: Effect of a *lacZ* inverted repeat on *lacZ* gene expression.

To further investigate the ability of dsRNA to inhibit gene expression in fission

15   yeast we generated a vector containing the full-length 3.5 kb frameshifted *lacZ* sequence with a 2.5 kb inverted repeat. This construct generates a transcript of approximately 7 kb in length with 2.5 kb of self-complementarity which, predictably, will form a strong intramolecular RNA duplex. This gene was initially integrated into a fission yeast strain in single copy and then crossed with the strain RB3-2. The resulting strain which

20   contained the single copy inverted repeat gene and target *lacZ* showed no reduction in β−galactosidase activity when transcription of the inverted repeat was activated. Southern analysis confirmed that the cassette was intact (data not shown) while RNA analysis indicated that the 7 kb transcript was being generated but approximately 10-fold less than episomally expressed antisense *lacZ* (Fig. 4A&B). To increase the steady-

25   state level of the inverted repeat RNA we generated the episomal plasmid pM53-1 containing this cassette. RNA analysis demonstrated that this vector is expressed at a similar level to episomally expressed antisense *lacZ* (Fig. 4A). When expressed in RB3-2 pM53-1 inhibited β−galactosidase activity by 40% (Fig. 2C), while addition of thiamine to the culture medium returned β−galactosidase activity to control leve.

30   To confirm that gene inhibition was due to this construct forming an RNA duplex, an in vivo assay for dsRNA was developed. To this end, a series of vectors were generated which contained functional *lacZ* sequences including *lacZ* alone (pM85-1), a

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lacZ inverted repeat (pM81-2), and the *lacZ* repeat with both sequences in the sense orientation (pM91-1) (Fig. 5A). These vectors were then introduced into a strain lacking the integrated target sequence (NCYC2037; h^+, *ura4-D18*) and resulting transformants were overlayed with X-GAL-containing agarose. Both strains containing control vectors

5   generated high levels of β–galactosidase while the strain expressing the *lacZ* inverted repeat did not (Fig. 5B). In each case Northern analysis demonstrated that strains were expressing the transgene. These results indicate that the inverted repeat RNA could not be efficiently translated to produce β–galactosidase, most probably due to the formation of a strong intramolecular hairpin structure. Taken together, these results show that a

10   construct which has the ability to form dsRNA by intramolecular hybridization will interfere with target gene expression when expressed at high levels, again indicating that dsRNA mediates target gene suppression in a dose-dependent manner in fission yeast.

EXAMPLE 5: Gene-specificity of dsRNA-meditated gene silencing in fission yeast.

To test the ability of dsRNA to specifically interfere with other target sequences

15   in fission yeast, sense and antisense *c-myc* sequences were co-expressed in a strain containing an integrated *c-myc-lacZ* fusion cassette (Fig. 1D; (3)). A 792bp antisense *c-myc* fragment from exon 2 of the human *c-myc* gene (named CM-17) was previously found to suppress β-galactosidase activity within a *c-myc-lacZ* fusion target strain (AML1) by 47% (3). CM-17 was subsequently subcloned into pREP4 in the sense

20   orientation to generate pN12-1. The antisense *c-myc* vector (pCM-17) and the sense *c-myc* vector were transformed into AML1 both independently and together. β-galactosidase assays demonstrated that co-expression of the antisense and sense constructs enhanced *c-myc* suppression by an additional 13% compared with the antisense *c-myc* vector alone (Fig. 2D). Transformation of RB3-2 with the antisense and

25   sense *c-myc* constructs resulted in no down-regulation of β-galactosidase activity indicating that the action of dsRNA is gene-specific (Fig. 2D). These results demonstrate that dsRNA can specifically interfere with multiple targets in fission yeast including one of human origin.

EXAMPLE 6: Over-expression of a translation factor can enhance RNAi in fission

30   **yeast.**

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The dose-dependency of dsRNA-mediated gene silencing in fission yeast described above has allowed us to use an over-expression strategy to identify genes involved in RNAi. In comparison to mutagenesis strategies, over-expression can enable the identification of genes which are otherwise essential for cell viability. Also, cellular

5   factors that quantitatively enhance or reduce RNAi activity can be determined. The first gene that we have tested in mediating RNAi activity in the present model has been the *S. pombe* ATP-dependent RNA helicase gene, *ded1*. *Ded1* is an essential gene which has previously been characterized as a suppressor of sterility, a suppressor of checkpoint and stress response, and a general translation initiation factor. According to current

10   models of dsRNA-mediated gene regulation an ATP-dependent RNA helicase may be required in conjunction with a dsRNA-dependent RNA polymerase for the formation of short single-stranded RNA fragments which specifically degrades target mRNA. We therefore reasoned that over-expression of this gene in fission yeast could enhance the efficiency of dsRNA-mediated gene silencing by stimulating the unwinding of dsRNA.

15   Co-expression of the *ded1* vector with the antisense *lacZ* vector significantly enhanced dsRNA-mediated *lacZ* inhibition by a further 50% compared to the antisense expressing strain (Fig. 6A). When *ded1* was over-expressed in the absence of the antisense *lacZ* vector, β–galactosidase activity was comparable to control strains indicating that its effect was dependent on the presence of dsRNA (Fig. 6A). The *ded1* vector was also co-

20   expressed with a short antisense *lacZ* vector which has previously been shown to be less effective than the full-length antisense gene (2). Again, the over-expression of *ded1* stimulated dsRNA-mediated *lacZ* inhibition (Fig. 6B). We have thus shown that over-expression of an ATP-dependent RNA helicase greatly enhances RNAi in fission yeast. Although an ATP-dependent RNA helicase has recently been suggested to be a key

25   component in RNAi, this is the first demonstration of its intrinsic involvement. Further, it can clearly be rate limiting, as the over-expression leads to increased RNAi activity in this system. This ability of the *ded1*-encoded RNA helicase is consistent with its activities as a member of the DEAD box family of helicases, with their three core domains of ATPase, RNA helicase, and RNA binding activities. These could allow the

30   enzyme to enhance gene suppression as follows: (i) in a dissociative mechanism it could mediate either the unwinding of dsRNA to generate a cRNA in conjunction with an RNA-dependent RNA polymerase or strand separation of fragmented dsRNA to

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enhance binding to homologous transcripts, and/or (ii) in an associative mechanism it could catalyse the ATP-dependent exchange of the sense strand of the short dsRNA with the target mRNA. The presence of *ded1* homologues in other organisms displaying RNAi further supports the general involvement of this component in the RNAi

5   machinery.

EXAMPLE 7: Over-expression of a transcription factor can enhance RNAi in fission yeast.

The second gene investigated was the *nmt1* transcription factor *thi1*. This gene has been shown to specifically up regulate *nmt1* expression when overexpressed in

10   fission yeast. As we have previously shown that antisense RNA-mediated gene suppression is dose-dependent in *S. pombe* (1) it was hypothesized that over-expression of *thi1* would result in increased production of *nmt1*-driven antisense *lacZ* RNA and a consequent enhancement in target gene suppression. The *thi1* open reading frame was PCR-amplified and subcloned into pREP4 as a *Bam*HI fragment. This vector was then

15   transformed into RB3-2/pGT2 and β-galactosidase assays performed. As predicted, *lacZ* suppression was enhanced when compared to a strain expressing antisense RNA alone (Fig. 7). This result indicated that host-encoded factors are present which modulate the efficacy of dsRNA-mediated gene suppression in fission yeast and also validated the over-expression strategy.

20   **EXAMPLE 8: Screening for novel RNAi modulating factors.**

Over-expression of a fission yeast cDNA library (5) in an antisense *lacZ* expressing strain, revealed a series of transformants in which β-galactosidase activity was significantly reduced from that demonstrated in antisense RNA-expressing strains alone. The screening strategy is shown in figure 8. Transformants were grown on

25   selective plates and overlayed with X-GAL-containing media to generate a blue phenotype which could be analysed visually in a semi-quantitative manner (Fig. 9A)(3). From 12,000 transformants screened, 48 were initially identified as having a reduced blue phenotype compared with background transformants. Of these, 25 demonstrated a reproducible enhancement in antisense RNA-mediated *lacZ* suppression when

30   quantified by a liquid β-galactosidase assay (Fig. 9B). To demonstrate that the observed effects were due to transcription of the cDNA insert, transformants were grown in the

presence of thiamine. This repression of the *nmt1* promoter resulted in a return to control levels of β-galactosidase activity in all transformants (Fig. 9B). This indicated that the enhanced suppression was due to expression of the cDNA rather than other genetic events such as *lacZ* reporter mutations. To determine if the effect was dependent

5 on the presence of dsRNA, the antisense plasmid was segregated out of the transformants and the strains were again assayed for β-galactosidase activity. Nine of the 25 transformants demonstrated a return to the level of β-galactosidase seen in the parental strain indicating that the effect was dsRNA-dependent (Fig. 9B). The cDNAs being expressed in these transformants were named *R*NAi *e*nhancing *s*equences (*res*).

10 All transformants displayed normal growth phenotypes indicating that over-expression of the resident cDNAs did not affect general biology of the cell. In the absence of antisense RNA the remaining 16 transformants retained a reduced β-galactosidase activity indicating that the enhanced gene silencing was not dependent on the presence of dsRNA. This suggests that the cDNA-encoded proteins in these strains could have

15 been affecting *lacZ* at the level of transcription or the stability or function of the protein.

The library plasmids were recovered from the *aes*-containing strains (also referre to as *res*-containing strains) and their cDNA inserts sequenced. BLASTN and BLASTP analysis identified clones W18, W20, and W30 (named *aes2*) as homologues of domains 2 and 3 of the mitochondrial elongation factor Tu (EF Tu). EF Tu is an

20 essential protein which plays a role in transporting tRNA to the A site in the ribosome for peptide elongation. The cDNA in transformants W21, W23, and W32 (named *aes3*) was homologous to a putative protein that was identified in a screen for fission yeast ORFs. Interestingly, the cDNA insert was also homologous to the antisense strand of the 3' UTR of the fission yeast gene sna41 which has previously been shown to be

25 involved in DNA replication. It is thus possible that *aes3* may operate through more than one mechanism in enhancing antisense RNA activity. The cDNA in transformant W47 (named *aes4*) was homologous to the antisense strand of the ribosomal protein L7a, a component of the 60S ribosomal subunit. aes4 also contained a small ORF of unknown biological function. The inserts in transformants W27 and W28 (named *aes1*)

30 shared homology with a putative protein from *C. albicans* that was identified in a screen for genes essential for cell growth. A tertiary quantitative β-galactosidase assay was performed to obtain accurate levels of gene silencing augmentation in the antisense lacZ

strains co-expressing these unique factors (Fig. 9C). This analysis showed that these co-factors enhanced antisense suppression by up to 50%. These results demonstrated that over-expression of a cDNA library was an effective way of identifying novel co-factors that magnify the suppressive effect mediated by antisense RNA.

5   *aes1* shared 43% identity with amino acids 4 to 202 of a *C. albicans* hypothetical protein (AJ390519). *aes2* shared 99% identity with nucleotides 10452 to 9484 of the translation elongation factor EF Tu (AL049769). *aes3* shared 94% identity with nucleotides 776 to 1145 of D89239 *S. pombe* ORF (D89239) and 93% identity with nucleotides 3246 to 2876 of the antisense strand of the DNA replication factor sna41

10   (AB001379). *aes3* also contained a 220 nt stretch of a GA repeat sequence at its 3' end. *aes4* shared 99% identity with nucleotides 9678 to 8897 of the antisense strand of ribosomal protein L7a (AJ001133) and 99% identity with nucleoStides 1365 to 584 of the antisense strand of ribosomal protein L4 (AB005750). The reference numerals in brackets refer to accession numbers in the GeneBank database (the GeneBank database can be

15   accessed from the following web site: http://www.ncbi.nlm.nih.gov/).

EF Tu is analogous to the eukaryotic EF1α and acts by transporting tRNA to the A site in the ribosome for peptide elongation. EF1α is an essential protein which has also been implicated in a large array of cellular activities including actin binding, microtubule severing, cellular transformation, cell senescence, protein ubiquitination,

20   and protein folding. Detailed analysis of the EF Tu-expressing strain showed that it enhanced antisense RNA-mediated *lacZ* silencing by an additional 15% (Fig. 9C). It has been proposed that dsRNA is fragmented into 21-25 nt species by dsRNA-specific nucleases, amplified by RNA-dependent RNA polymerase, and dissociated by an ATP-dependent RNA helicase. The small antisense fragments are then free to attack

25   homologous mRNA by RNA nuclease-mediated degradation. It has recently been suggested that an associative mechanism could catalyse the ATP-dependent exchange of the sense strand of the short dsRNA with the target mRNA. This may involve the transport of fragmented dsRNA to the ribosome where, in a competitive reaction with tRNA complexes, the complementary antisense strand binds to the mRNA. This would

30   permit the association of the antisense strand of the dsRNA with the target mRNA when the latter is in a structurally exposed state and both inhibit translation and target the mRNA for ribonuclease degradation. Without wishing to be bound by any particular

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mechanism of action, EF Tu could act by binding to RNAi-dependent short dsRNA species and bring them to the site of action.

The protein encoded by *sna41* has previously been shown to be involved in DNA replication. *sna41* has low homology with *CDC45* and might have DNA helicase

5   properties which could facilitate the expression of complementary sequences. It is conceivable that the antisense plasmid and target DNA sequences may ectopically pair by intermolecular complementarity. Such pairing may inhibit RNA expression and consequently reduce the level of intracellular dsRNA. Similarly the intramolecular pairing of inverted repeat DNA sequences may also interfere with RNA expression. The

10   over-expression of a protein with DNA helicase properties could facilitate the generation of more dsRNA which could in turn enhance the RNAi effect. Furthermore, *CDC45* mutants show an increased rate of plasmid segregation. If plasmid loss is inhibited by over-expression of *sna41* then more dsRNA may be generated leading to more effective RNAi in this system. In this light it is not unreasonable to expect that

15   other proteins normally involved in DNA and/or RNA metabolism and function could also have a role in RNAi modulation and/or enhancement.

The L7a protein is part of the 60s ribosomal sub-unit. Without wishing to be bound by any particular mechanism of action, RNAi augmentation by over-expression of this protein is reasonable as it is hypothesised that the short dsRNA species may

20   undergo strand displacement with target mRNA at the ribosome. The L7a ribosomal protein may act in RNAi by i) mediating docking of dsRNA or its unwound form into the A site of the ribosome, ii) assisting in association of the antisense strand with the target mRNA, and/or iii) shuttling of dsRNA to the ribosomal complex.

Nucleotide sequences representative of the *aes* factors referred to above are

25   provided below and identified as Seq ID Nos 1 to 4 :

DNA sequence for *aes1* factor (Seq ID No 1)

```
TTTAACTTAGTTCGCTTTGTTAAATTGGCCTCGAGGTCGACGTTAATTAAGCCGCAATT
GTAACAGTAGATTTTTTGCATCATTATTACTCTCCGAAACATGACTGAACACTCATTTA
AGCAAATAGACGTGTTTTCTAATAAAGGTTTTCGAGGTAATCCTGTTGCAGTTTTTTTT
GATGCAGATAATTTATCACAAAAGGAAATGCAGCAGATTGCCAAGTGGACAAATTTATC
TGAGACAACATTTGTTCAAAAGCCGACAATCGATAAAGCAGATTACAGACTTCGTATAT
TTACCCCAGAATGTGAATTAAGCTTTGCTGGTCACCCAACAATTGGATCGTGCTTTGCT
GTTGTTGAAAGTGGATATTGTACTCCAAAAAACTGTAAAATTATTCAGGAATGTTTAGC
CGGTTTAGTTGAATTAACTATCGATGGGGAAAAGGATGAAGACACTTGGATTTCTTTCA
AACTTCCGTATTACAAAATTTTACAGACTTCTGAAACTGCAATTTCAGAAGTAGAAAAT
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GCATTGGGTATTCCTCTGAATTATAGTTCTCAAGTTTCTCCTCCTGTGTTAATAGATGA
TGGACCAAAGTGGCTTGTAATTCAACTTCCAAACGCTACAGATGTGCTCAACCTCGTTC
CGAAATTTCAGTCCCTTTCCCAAGTTTGTAAAAACAATGATTGGATAGGCGTCACCCGT
CTTTGGTGAATTAGAAAAGACTCGTTTGAAAGCCCGAAGCTTTGCGCCTTTAATACATG

5   TCAATGAGGATCCGGCTTGCGGTAGTGGTGCAGGAGCTGTCGGTGTGTATATTGGAAGC
TCTCAAAAAACTCCAACTTCTCTATCATTTACGATTTCTCAAGGTACAAAATTAAGTAG
ACAAGCAATTTCCAAAGTCAGCGTAGACGTTTCCTCCAATAAATCAATTGCTGTTTTTG
TCGGTGGACAGGCAAAAACTTGTATTTCTGGAAATCGTTTATTTAATGTTTTTATTAC
AAATATTCACTTGCGAGTTTATTTTCCAATACTGAAGACTTTCAATCAATAGCAAATAT

10   GCTACTCAAGGAAGTTCACTCATTCAAAAGCAATTGGTTTACTATATCGTTTTTTCTAA
CTAGTTACTAGTCATTGAACAATCTACCGAATGATAAAATGAAATTTTGGTTTTTCCCC
GGGTAAAAGGAATGTCTCCCTTGCCAGTACTGCTAGGGTTTTTCTTTCGAACTATAAGA

DNA sequence for *aes2* factor (Seq ID No 2)

15   AGTCCGCTTTGTTAAATTGGCCTCGAGGTCGACGTTAATTAAGCCTGATATGATCGAGC
TTGTCGAAATGGAAATGCGTGAGCTACTCTCCGAATACGGATTTGATGGTGACAATACT
CCAATTGTTAGCGGCAGTGCTTTATGTGCCTTAGAGGGTCGTGAGCCTGAGATTGGTCT
CAATAGTATTACTAAATTGATGGAAGCTGTTGATAGTTATATTACTCTTCCTGAAAGAA
AAACGGATGTCCCTTTCTTGATGGCCATCGAGGACGTTTTTTCAATTTCAGGTCGCGGA

20   ACTGTAGTCACTGGCCGTGTCGAGCGCGGTACTTTAAAGAAGGGTGCTGAAATCGAAAT
CGTCGGTTATGGTAGCCATTTAAAGACTACCGTTACTGGAATTGAAATGTTCAAAAAGC
AGCTTGATGCCGCCGTTGCCGGTGACAATTGTGGCCTTTTACTTCGTTCTATCAAGCGA
GAGCAATTAAAACGTGGAATGATTGTCGCTCAACCAGGAACCGTTGCTCCTCATCAGAA
ATTCAAGGCATCATTCTATATTTTGACAAAGAGGAAGGAGGTCGTCGTACCCGGTTTC

25   GTTGACAAGTATCGTCCCCAACTGTACAGTCCGTACTTCGACGTTACTGTCGAACTTA
CCCACCCTGATCCTAACGACTCAACAAAATGGTTATGCCTGGAGACAATGTCGAGATGA
TCTGTACGCTTATTCACCCCATTGTCATCGAAAAAGGACAACGCTTCACAGTTCGTGAG
GGTGGAAGCACTGTAGGCACAGCTTTGGTTACTGAACTTTTGGATTAGTGCATTTATGA
ACTTATTGGCTTTAAAAATTTTGCATGCTGAATACCAATATTATGTCCCTTCTCAGAAT

30   TCTATAACTACAGTGTCATTATTGTAATAAGACTTTTGCATCCATTGACAATGGTATTT
GATACTTTTATAGTTTCTACTATTGTTAGCCAAAGTTATAAAACAAATAATAAAATAAC
GTTGAATCAAAAAAAAAAAAAAAAAAAAGCGGCCGCGGATCCCCGGGTAAAAGGAATGTC
TCCCTTGCCAGTACTGCTAGGGTTTTTCTTTCAAACTATGGGA

DNA sequence for *aes3* factor (Seq ID No 3)

35   ATTTCAGACGCAATTCACATGGCTTTTGACTGTATTGCTATTCTTGTCGGTTTAGTTGC
TACGACGCTTGCCAAGATGCCTCTAAATTATGCTTACCCCTTTGGATTTGCAAAAATTG
AGGCTCTTTCGGGTTTCACTAATGGTATTTTTTTAGTTTTGATTTCATTTTCTATCGTC
GGCGAGGCATTATATAGGTTATTTCATCCGCCCCAAATGAATACCGACCAATTGTTGTT
GGTTAGTTTTTTGGGCCTTGTTGTGAATTTGGTAGGTATCCTAGCGTTCAATCATGGGC

40   ATAATCATGATCATGGGTCTCATCACCATCATTCCCATAGTAATCATAGTATGTGTCTG
CCTAACACTACAAATGATATAAATATTTTTGAAGAGTTTGAAGAAGAAAAGATAATGT
TGAAGCCCAGAAAATGGGCTATACGAATGACGATCACGTATCCCAACATGAACATACCC
ATGAGAATAGTCAGGAACATCACCATGAGCATAACCACAATCATGATCACATCCATAAA
TACAATGAAAAATGCGACCATGAAAGCATAAGTCTCCAGAATTTAGACAATGATCATCA

45   CTGTCATCATCACCATGAAAATCATAATATGCATGGCATATTTCTGCATATTATCGCAG
ATACTATGGGCTCTGTTGGAGTTATTGTCTCTACTATATTAATACAGTGGTTTTCATGG
ACCGGTTTTGATCCTTCGGCATCTCTAATAATTGCTGCATTAATATTTGTTTCTGTACT
TCCATTAATTAAAGATTCGGCGAAGAATTTGCTCTCTGTGACTGATCCAGAATCGGAAT

ATTTATTGAAGCAGTGTTTGTCGAACATCAGTTTAAGTCACTCCGTTGTCAGTTTATCC
AACCCTAAGTTCTGGACAAACGAAAGAGGTGAAGTGTATGGAATACTCCATATTCAGGT
GAGCATAGACGGTGATTTAAACGTGGTTCGTAATGAAGTATTTAGGAAGCTCTCAATCG
CTGTACCAAATTTAAAACACATTTGTATACAATCTGAACGGCCAAACAATTGCTGGTGT
5   GGAAAATAGTTCTTACATCAGTTGATATCCATACTTATTTACGTGTAATTTTAATTAGA
TGAATTAATATTTTCTTTATTAGC

DNA sequence for *aes4* factor (Seq ID No 4)

TTTACTTTAGTCGCTTTGTTAAATTGGCCTCGAGGTCGACGTTAATTAAGCTTTTTTTT
TAAGAGATATAACATATGTCAACGCGTCATTGATTAACTACATAACACGCCAATTATAA
10   ACTTCTCCCAAAAGAACTTAAGAATTTCCATTTTCAATCCAGATGAATTTATTTAAGAG
ACGAACAGTAGCGGCAGCAGCCTTAGCACGCTTAGCGAGCAAAGCTTGGGTCTTACCAC
CCATGATACCCACCACCCCACTTACGACGGGCTTTCGTCGTACTTAGCAGAGAAGTTAG
CATCAACGGCGGAGACAATAGAAGCGAGTTCGTTCTTGTCTTCCTCACGGACCTCAGTG
ACAGCTAAAACAGCAGCAGTCTTTTGGTGAATGACAGTACCAAGGCGGGCCTTGTTCTT
15   GACAATGGCATAAGGAACACCCATCTTCTTGCACAAAGCAGGCAAGAAAACGACGAGTT
CAATGGGGTCGACATCGCTGGCAATGAGAACCAACTTAGCCTTCTTGGCCTCAATGAGA
GCTACAACATGGTTCAAACCATATTTAACATTGTAAGGCTTCTTAGAGACGTCTTGAGC
AGACTTGCCGTTGGCAACAGCCTCGGCTTCAGCAACCAAACGTTGCTTCTTTTCAGCAG
CAGTCTCAGGACGGTACTTGTTAAGCAACTTGAAGACCTGAGTAGCAGTGTTTTTGTCC
20   AAAGTCTTCTGGAACTGAGCAATGGCAGGAGGAACCTTCAAACGCAAGTTCAAAATCTT
GCGACGGCGTTGAAGGCGGATATACTCAGGCCACTTAACAAAACGGCTCAAGTCACGCT
TAGGTTGGATGTCTTGTCCCCCGGGTAAAAGGAATGTCTCCCTTGCCAGTACTGCTAGG
GTTTTTCGTTCGAATAAGGCC

EXAMPLE 9. Effect of novel RNAi factors on antisense RNA and dsRNA-

25   **mediated gene regulation**

With recent studies on PTGS suggesting that antisense RNA, co-suppression,

and dsRNA-mediated interference may share similar mechanisms, we wanted to

determine whether over-expression of an aes factor would also enhance dsRNA-

mediated regulation. Having demonstrated that dsRNA could mediate gene suppression

30   in this fission yeast model, the effect of an antisense enhancing sequence on dsRNA-

mediated regulation was tested. To this end, the aes2 gene was co-expressed with the

lacZ panhandle construct in a yeast strain containing the lacZ target gene. Under these

conditions, this transformant displayed an additional 30% suppression of β-

galactosidase activity when compared to the transformant expressing only the panhandle

35   lacZ RNA (see Figure 10). This result shows that the aes2 gene, encoding the two

domains of the mitochondrial elongation factor EF Tu, stimulated not only antisense

RNA- but also dsRNA-mediated gene inhibition.

Further it was shown that these two forms of regulation are related by over-

expressing the ATP-dependent RNA helicase ded1 in the presence of lacZ antisense

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RNA and showing that this helicase enhanced gene suppression by a further 50% compared to the control strain (example 6). ded1 was tested on both active and inactive antisense plasmids and demonstrated that ded1 augmentation of gene silencing was dependent on an active antisense RNA (see Figure 11). This could be due to the absence

5   of RNA duplex formation with the inactive antisense RNA and the consequent lack of a substrate for the RNA helicase.

The methods of the present invention have utility in demonstrating a range of RNAi efficacies, in identifying new factors which enhance or reduce gene silencing, in inhibiting gene expression or increasing sensitivity to antisense inhibition of gene

10 expression, in the treatment or prevention of disorders which require inhibition or down-regulation of gene expression.

Although the present invention has been described with reference to specific examples and preferred embodiments it will be clear to those skilled in the art that variations and modifications which do not depart from the concept and the spirit of the

15 invention described herein are also contemplated as being within the scope of the present invention.

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CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1.   A method for inhibiting the expression of a target nucleic acid in a cell, which method comprises the steps of

(i)   elevating in the cell the level of an RNAi factor, and

(ii)   prior, concurrently with or subsequent to performing step (i), introducing into the cell a molecule which is, or gives rise to, an anti-sense nucleic acid directed toward at least a portion of the RNA transcript of the target nucleic acid, under conditions permitting the RNAi factor to increase the degree to which the anti-sense nucleic acid inhibits expression of the target nucleic acid.

2.   A method of increasing cellular susceptibility to anti-sense-mediated inhibition of target nucleic acid expression, which method comprises elevating the level of an RNAi factor in a cell that expresses said target nucleic acid, with the proviso that the cell is to have prior, concurrently or subsequently introduced thereinto a molecule which is, or gives rise to, an anti-sense nucleic acid directed toward at least a portion of the RNA transcript of the target nucleic acid under conditions permitting the RNAi factor to increase the degree to which the anti-sense nucleic acid inhibits expression of the target nucleic acid.

3.   A method for treating a subject suffering from a disorder whose alleviation is mediated by inhibiting the expression of a target nucleic acid, which method comprises the steps of

(i)   elevating the level of an RNAi factor in the subject's cells where the target nucleic acid is expressed, and

(ii)   prior, concurrently with or subsequent to performing step (i), introducing into such cells a molecule which is, or gives rise to, an anti-sense nucleic acid directed toward at least a portion of the RNA transcript of the target nucleic acid, under conditions permitting the RNAi factor to increase the degree to which the anti-sense nucleic acid inhibits expression of the target nucleic acid, thereby treating the subject.

4.   A method for inhibiting in a subject the onset of a disorder whose alleviation is mediated by inhibiting the expression of a target nucleic acid, which method comprises the steps of

(i)   elevating the level of an RNAi factor in the subject's cells where the target nucleic acid would be expressed if the subject were suffering from the disorder, and

(ii)   prior, concurrently with or subsequent to performing step (i), introducing into such cells a molecule which is, or gives rise to, an anti-sense nucleic acid directed toward at least a portion of the RNA transcript of the target nucleic acid, under conditions permitting the RNAi factor to increase the degree to which the anti-sense nucleic acid would inhibit expression of the target nucleic acid were such expression to occur, thereby inhibiting in the subject the onset of the disorder.

5.   A method of determining whether inhibiting the expression of a particular target nucleic acid or the activity of its product may alleviate a disorder, which method comprises the steps of

(i)   elevating the level of an RNAi factor in a cell whose phenotype correlates with that of a cell from a subject having the disorder;

(ii)   prior, concurrently with or subsequent to performing step (i), introducing into the cell a molecule which is, or gives rise to, an anti-sense nucleic acid directed toward at least a portion of the RNA transcript of the target nucleic acid under conditions permitting the RNAi factor to increase the degree to which the anti-sense nucleic acid inhibits expression of the target nucleic acid; and

(iii)   determining whether the cell's phenotype now correlates with that of a cell from a subject in whom the disorder has been alleviated or the disorder is not evident, thereby determining whether inhibiting the expression of the target nucleic acid or the activity of its product may alleviate the disorder.

6.   A method according to any one of claims 1 to 5, wherein the target nucleic acid is an exogenous nucleic acid or a part thereof.

7.   A method according to any one of claims 1 to 6, wherein the level of the RNAi factor is elevated by introducing into the cell additional copies of, or agents which give rise to, the RNAi factor.

8.   A method according to any one of claims 1 to 7, wherein the factor is selected from the group consisting of a gene, cDNA, RNA or a protein.

9.   A method according to any one of claims 1 to 8, wherein the factor is selected from the group consisting of a transcriptional activator of the antisense nucleic acid, a component of the RNAi machinery, a component of the DNA replication machinery and a component of translational machinery.

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10.   A method according to any one of claims 1 to 9, wherein the RNAi factor is an *res* sequence.

11.   A method according to claim 10, wherein the factor is selected from the group consisting of ATP-dependent RNA helicase (*ded1*), transcriptional factor *thi1*, DNA replication protein sna41, ribosomal protein L7a, elongation factor EF-Tu and *res1* as herein defined.

12.   A method according to claim 11, wherein the res sequence is obtainable from transformed cells designated herein W18, W20, W21, W23, W27, W28, W30, W32 and W47.

13.   A method according to claim 11, wherein the *res* sequence is represented by Seq ID Nos 1 to 4.

14.   A method according to any one of claims 1 to 13, wherein the cell is a eukaryotic cell.

15.   A method according to 14, wherein the eukaryotic cell is a mammalian.

16.   A method according to claim 1 or claim 2, wherein the cell is a *Schizosaccharomyces pombe* cell.

17.   A method according to any one of claims 1 to 16, wherein the antisense nucleic acid corresponds to a part of the target nucleic acid.

18.   A pharmaceutical composition for use in performing the method of any one of claims 2 to 17 comprising

(i)   an expressible nucleic acid encoding, or capable of increasing or decreasing the expression of, an RNAi factor;

(ii)   a nucleic acid encoding a molecule which is, or gives rise to, an anti-sense nucleic acid directed toward at least a portion of the RNA transcript of the target nucleic acid; and

(iii)   a pharmaceutically acceptable carrier,

wherein the nucleic acids of (i) and (ii) may be situated on the same or different molecules.

19.   A pharmaceutical composition for use in performing the method of any one of claims 2 to 17 comprising

(i)   an nucleic acid which is the target nucleic acid or a part thereof, or an expressible nucleic acid encoding a factor capable of elevating the intracellular level of the target nucleic acid;

(ii)   a nucleic acid encoding a molecule which is, or gives rise to, an anti-sense nucleic acid directed toward at least a portion of the RNA transcript of the target nucleic acid; and

(iii)   a pharmaceutically acceptable carrier,

5   wherein the nucleic acids of (i) and (ii) may be situated on the same or different molecules.

20.   A cell having increased susceptibility to anti-sense-mediated inhibition of a target nucleic acid expression, which cell (i) expresses a target nucleic acid and (ii) comprises an elevated level of an RNAi factor, with the proviso that the cell is to have

10   introduced thereinto a molecule which is, or gives rise to, an anti-sense nucleic acid directed toward at least a portion of the RNA transcript of the target nucleic acid under conditions permitting the RNAi factor to increase the degree to which the anti-sense nucleic acid inhibits expression of the target nucleic acid.

21.   A cell according to claim 20, wherein the cell is a eukaryotic cell.

15   22.   A cell according to claim 20 or claim 21, wherein the cell is a *Schizosaccharomyces pombe* cell.

23.   A method for inhibiting the expression of a target nucleic acid in a cell, which method comprises the steps of

(i)   augmenting the level of the target nucleic acid or a part thereof in the cell, and

20   (ii)   prior, concurrently with or subsequent to performing step (i), introducing into the cell a molecule which is, or gives rise to, an anti-sense nucleic acid directed toward at least a portion of the RNA transcript of said target nucleic acid, under conditions permitting an increase in the degree to which the anti-sense nucleic acid inhibits expression of said target nucleic acid.

25   24.   A method of increasing cellular susceptibility to anti-sense-mediated inhibition of a target nucleic acid expression, which method comprises augmenting the level of the target nucleic acid or a part thereof in a cell expressing the target nucleic acid, with the proviso that the cell is to have prior, concurrently or subsequently introduced thereinto a molecule which is, or gives rise to, an anti-sense nucleic acid directed toward at least a

30   portion of the RNA transcript of the target nucleic acid under conditions permitting the increase in the degree to which the anti-sense nucleic acid inhibits expression of the target nucleic acid.

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25.   A method for treating a subject suffering from a disorder whose alleviation is mediated by inhibiting the expression of a target nucleic acid, which method comprises the steps of

(i)   augmenting the level of said target nucleic acid or a part thereof in the subject's

5   cells where the target nucleic acid is expressed, and

(ii)   prior, concurrently with or subsequent to performing step (i), introducing into such cells a molecule which is, or gives rise to, an anti-sense nucleic acid directed toward at least a portion of the RNA transcript of the target nucleic acid, under conditions permitting an increase in the degree to which the anti-sense nucleic acid

10   inhibits expression of the target nucleic acid, thereby treating the subject.

26.   A method for treating a subject suffering from a disorder whose alleviation is mediated by inhibiting the expression of a target nucleic acid, which method comprises the steps of

(i)   augmenting the level of the target nucleic acid or a part thereof in the subject's

15   cells where the target nucleic acid is expressed, and

(ii)   prior, concurrently with or subsequent to performing step (i), introducing into such cells a molecule which is, or gives rise to, an anti-sense nucleic acid directed toward at least a portion of the RNA transcript of the target nucleic acid, under conditions permitting an increase in the degree to which the anti-sense nucleic acid

20   inhibits expression of the target nucleic acid, thereby treating the subject.

27.   A method for inhibiting in a subject the onset of a disorder whose alleviation is mediated by inhibiting the expression of a target nucleic acid, which method comprises the steps of

(i)   augmenting the level of the target nucleic acid or a part thereof in the subject's

25   cells where the target nucleic acid would be expressed if the subject were suffering from the disorder, and

(ii)   prior, concurrently with or subsequent to performing step (i), introducing into such cells a molecule which is, or gives rise to, an anti-sense nucleic acid directed toward at least a portion of the RNA transcript of the target nucleic acid, under

30   conditions permitting an increase in the degree to which the anti-sense nucleic acid would inhibit expression of the target nucleic acid were such expression to occur, thereby inhibiting in the subject the onset of the disorder.

28.   A method of determining whether inhibiting the expression of a particular target nucleic acid or the activity of its product may alleviate a disorder, which method comprises the steps of

(i)   augmenting the level of the target nucleic acid in a cell whose phenotype correlates with that of a cell from a subject having the disorder;

(ii)   prior, concurrently with or subsequent to performing step (i), introducing into the cell a molecule which is, or gives rise to, an anti-sense nucleic acid directed toward at least a portion of the RNA transcript of the target nucleic acid under conditions permitting an increase in the degree to which the anti-sense nucleic acid inhibits expression of the target nucleic acid; and

(iii)   determining whether the cell's phenotype now correlates with that of a cell from a subject in whom the disorder has been alleviated or the disorder is not evident, thereby determining whether inhibiting the expression of the target nucleic acid or the activity of its product may alleviate the disorder.

29.   A method according to any one of claims 23 to 28, wherein the target nucleic acid is an exogenous nucleic acid or a part thereof.

30.   A method according to any one of claims 23 to 29, wherein the level of the target nucleic acid is augmented by introducing into the cell additional copies of, or agents which are capable of inducing intracellular over-expression of, the target nucleic acid.

31.   A method according to any one of claims 23 to 30, wherein the cell is a eukaryotic cell.

32.   A method according to 31, wherein the eukaryotic cell is a mammalian.

33.   A method according to claim 23 or claim 24, wherein the cell is a *Schizosaccharomyces pombe* cell.

34.   A method according to any one of claims 23 to 33, wherein the antisense nucleic acid corresponds to a part of the target nucleic acid.

35.   A cell having increased susceptibility to anti-sense-mediated inhibition of a target nucleic acid expression, which cell (i) expresses said target nucleic acid and (ii) comprises an elevated level of said target nucleic acid, with the proviso that the cell is to have introduced thereinto a molecule which is, or gives rise to, an anti-sense nucleic acid directed toward at least a portion of the RNA transcript of the target nucleic acid

under conditions permitting the RNAi factor to increase the degree to which the anti-sense nucleic acid inhibits expression of the target nucleic acid.

36.   A cell according to claim 35, wherein the cell is a eukaryotic cell.

37.   A cell according to claim 35 or claim 36, wherein the cell is a *Schizosaccharomyces pombe* cell.

38.   Method of identifying a cellular factor capable of effecting and/or modulating expression of a target nucleic acid in a cell having the target nucleic acid and a nucleic acid which is an antisense of the target nucleic acid or part thereof, which method comprises over-expressing said factor in the cell and wherein the expression of the target nucleic acid is capable of being enhanced or only partially suppressed.

39.   A factor identified by the method of claim 38.

40.   A factor according to claim 39, wherein the factor is selected from the group consisting of a gene, cDNA, RNA or a protein.

41.   A factor according to claim 39 or claim 40, wherein the factor is selected from the group consisting of a transcriptional activator or the antisense nucleic acid, a component of the RNAi machinery, a component of the DNA replication machinery and a component of translational machinery.

42.   A factor according to any one of claims 39 to 41, wherein the factor is an *res* sequence.

43.   A factor according to claim 42, wherein the factor is selected from the group consisting of ATP-dependent RNA helicase (*ded1*), transcriptional factor *thi1*, DNA replication protein sna41, ribosomal protein L7a, elongation factor EF-Tu and *res1* as herein defined.

44.   An RNAi factor which is an *res* sequence obtainable from transformed cells designated herein W18, W20, W21, W23, W27, W28, W30, W32 and W47.
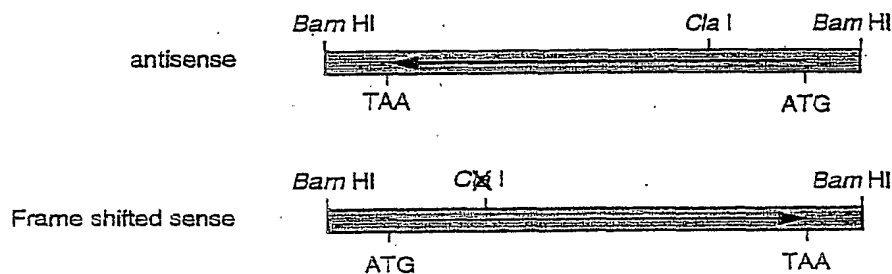
45.   An RNAi factor which is an *res* sequence represented by Seq ID Nos 1 to 4.

46.   A *Schizosaccharomyces pombe* cell having a target nucleic acid or a part thereof and a antisense nucleic acid or a part thereof which corresponds to the target nucleic acid or a part thereof, wherein the expression of the target nucleic acid is capable of being enhanced or only partially suppressed.

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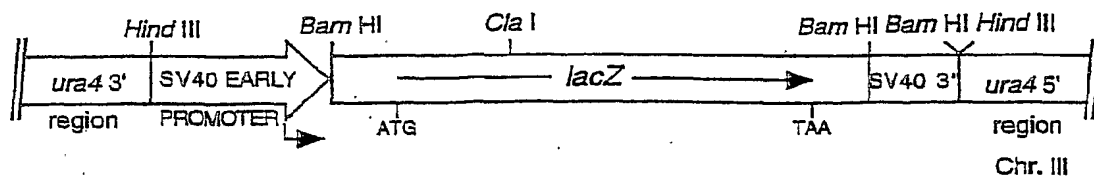
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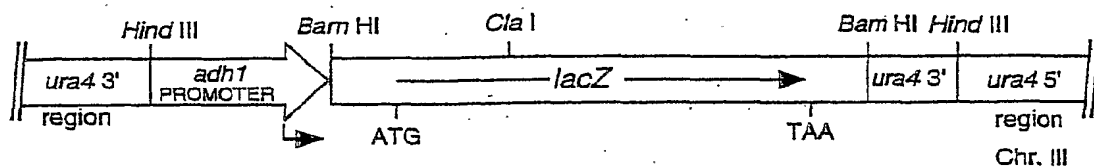
C *lacZ* antisense and sense fragments

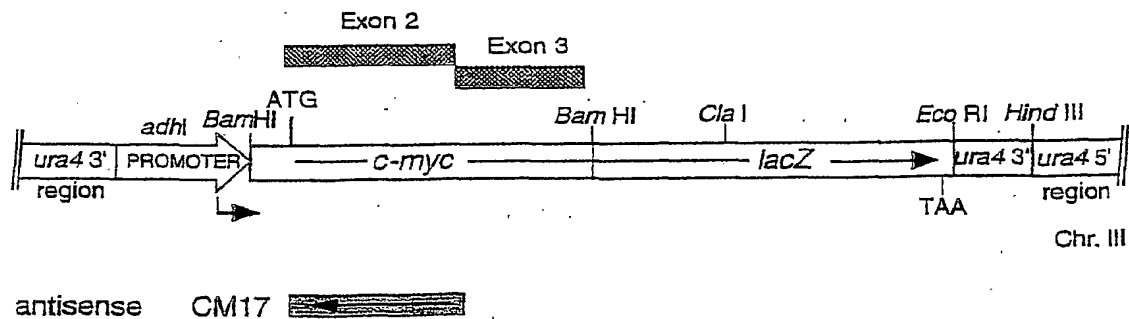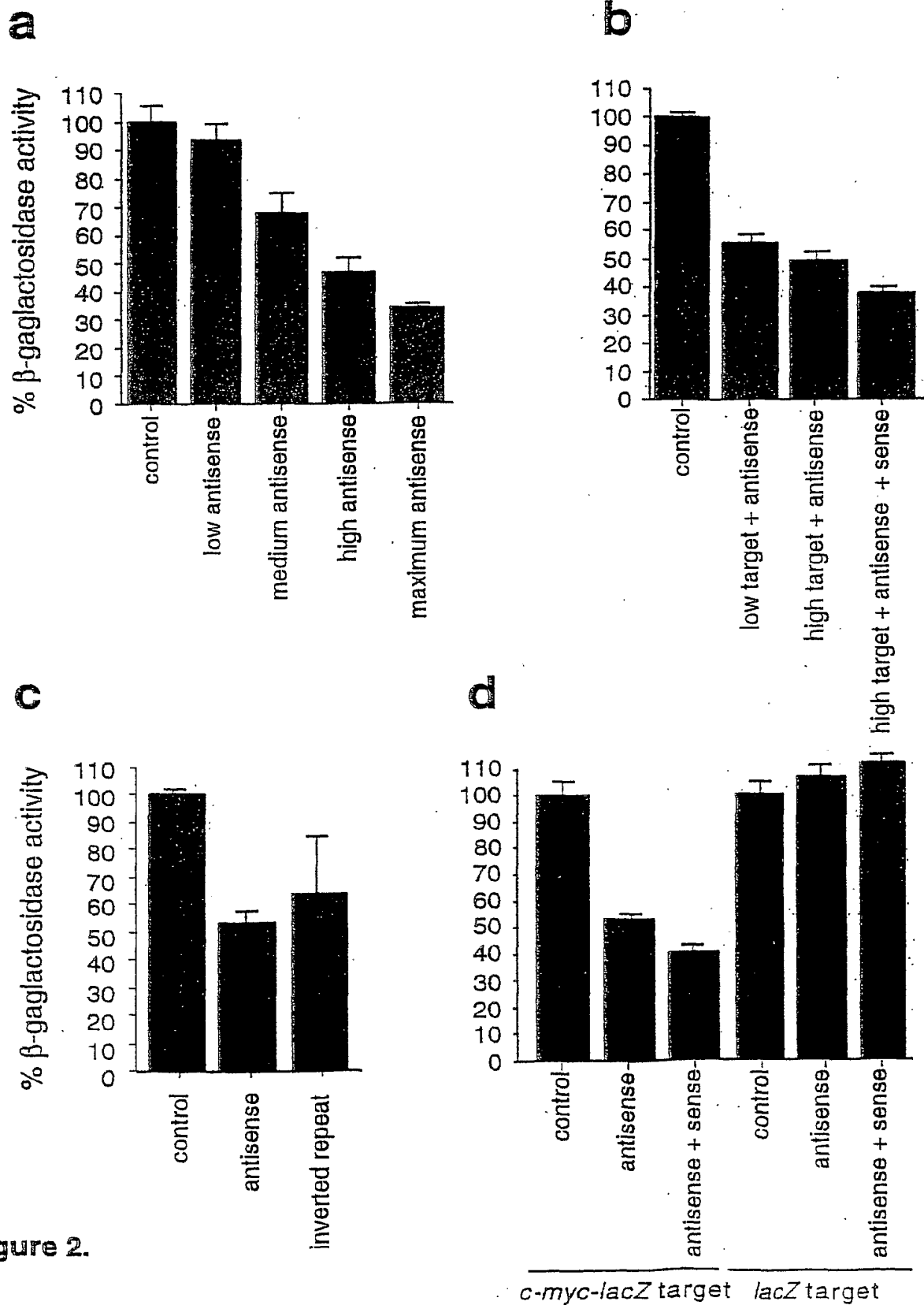**D** Target gene fusion: AML1 strain and antisense fragment
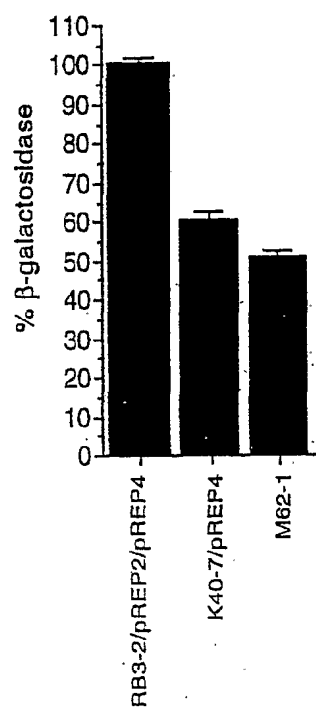

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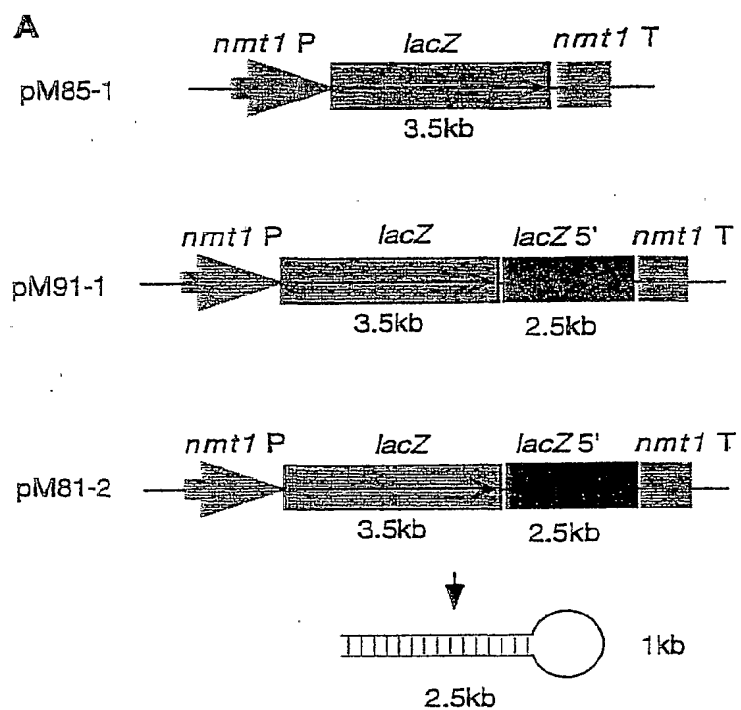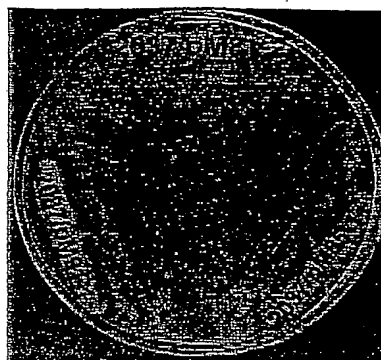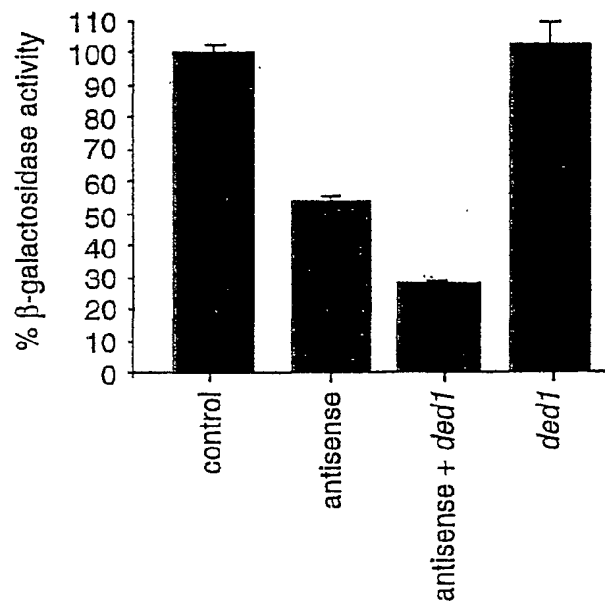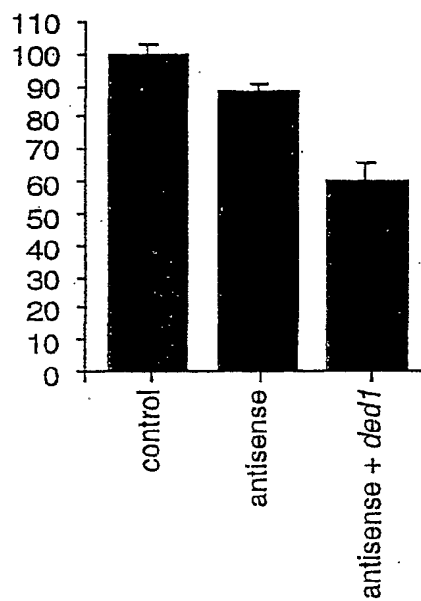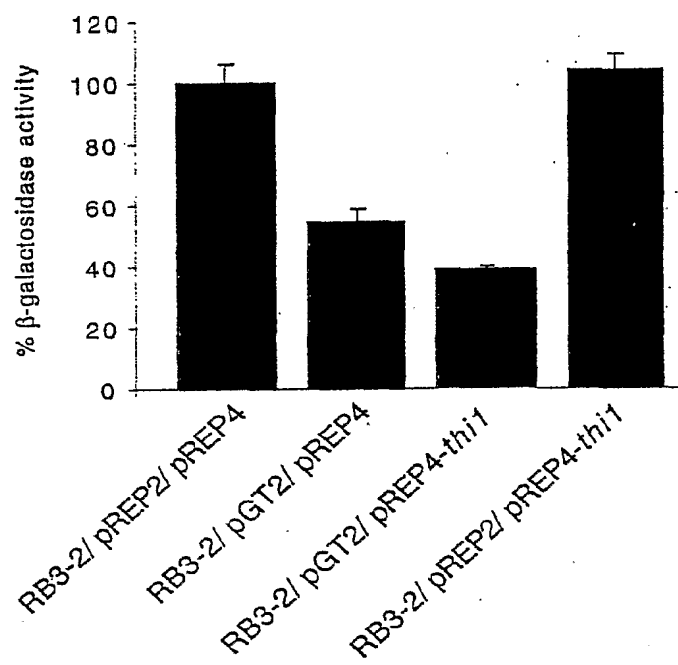
Figure 2.

Figure 3

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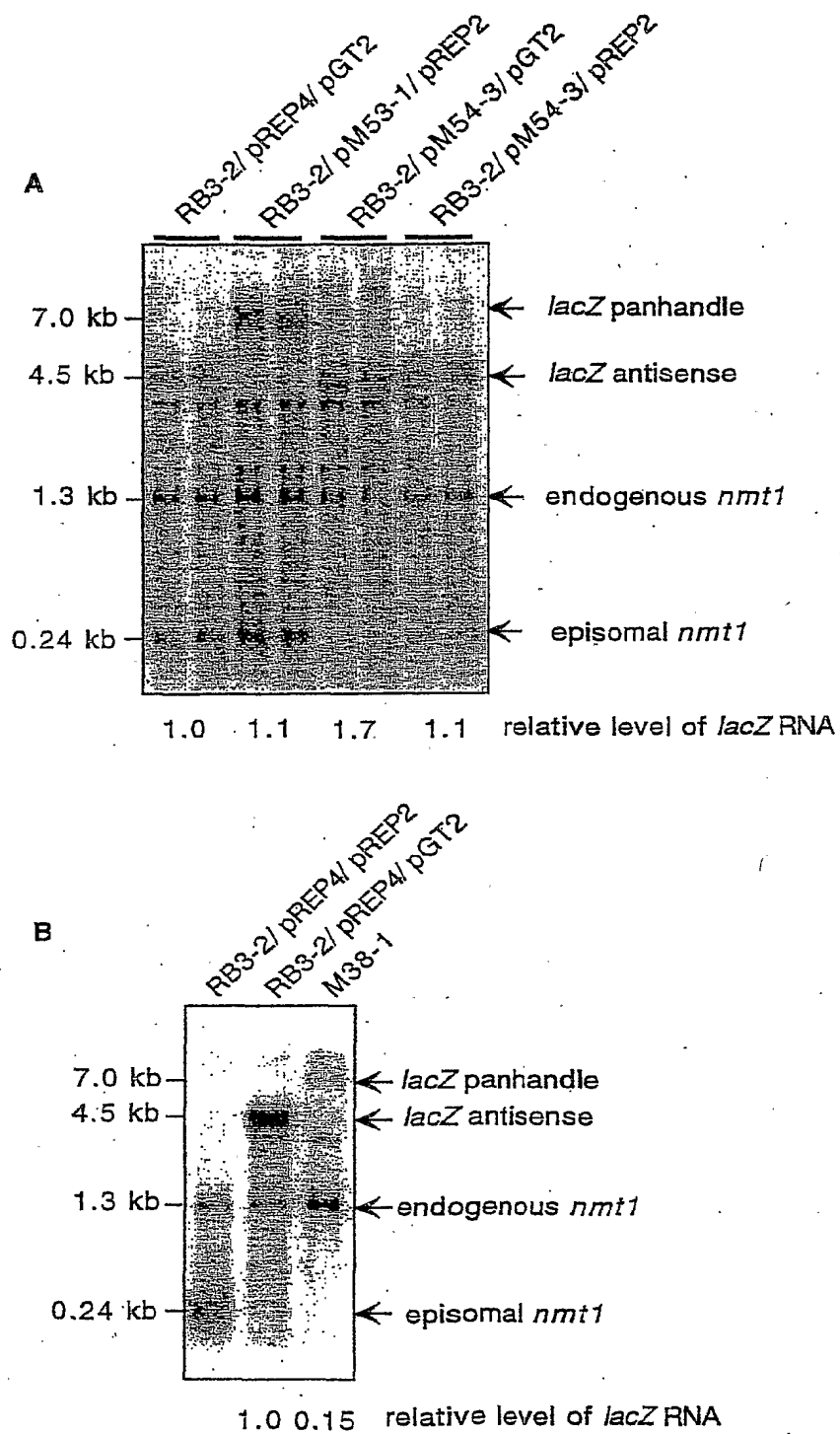RB3-2/ pREP4/ pGT2
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RB3-2/ pM54-3/ pGT2
RB3-2/ pM54-3/ pREP2

7.0 kb ← ← *lacZ* panhandle

4.5 kb ← ← *lacZ* antisense

1.3 kb ← ← endogenous *nmt1*

0.24 kb ← ← episomal *nmt1*

1.0   1.1   1.7   1.1   relative level of *lacZ* RNA

B

RB3-2/ pREP4/ pREP2
RB3-2/ pREP4/ pGT2
M38-1

7.0 kb ← ← *lacZ* panhandle
4.5 kb ← ← *lacZ* antisense

1.3 kb ← ← endogenous *nmt1*

0.24 kb ← ← episomal *nmt1*

1.0 0.15   relative level of *lacZ* RNA

Figure 4

Figure 5

a

b

Figure 6.

Figure 7

Figure 8.

a

b

c
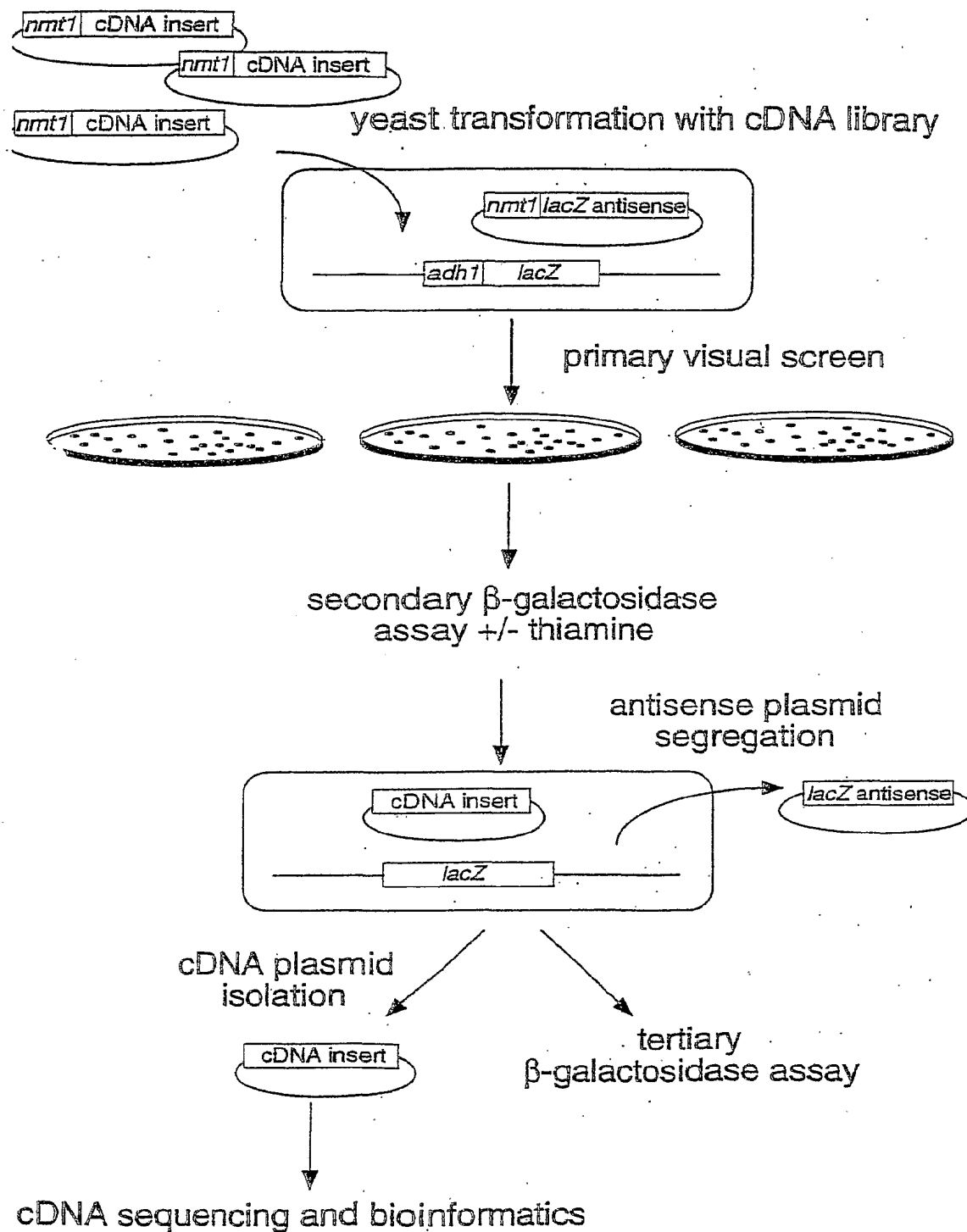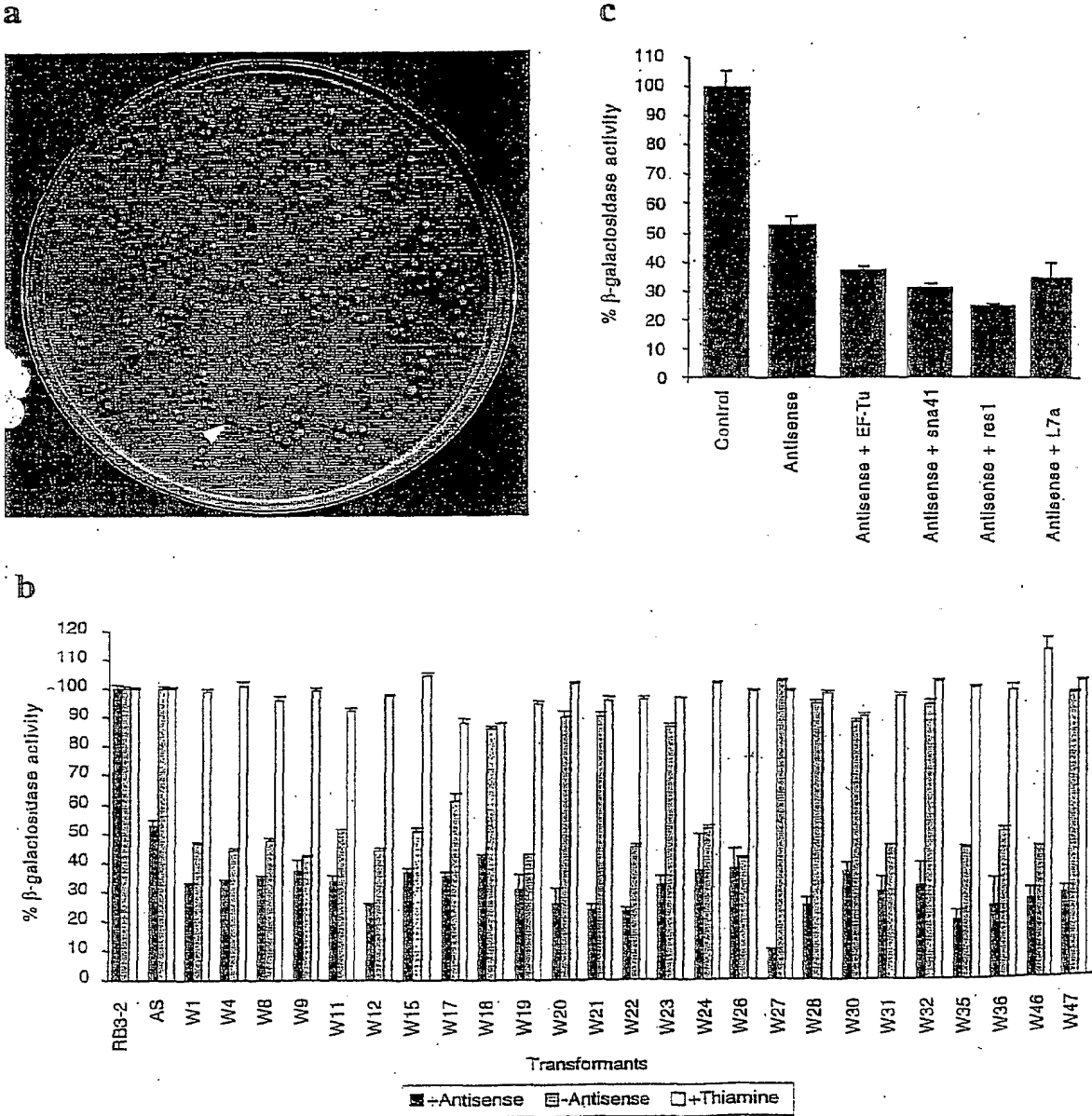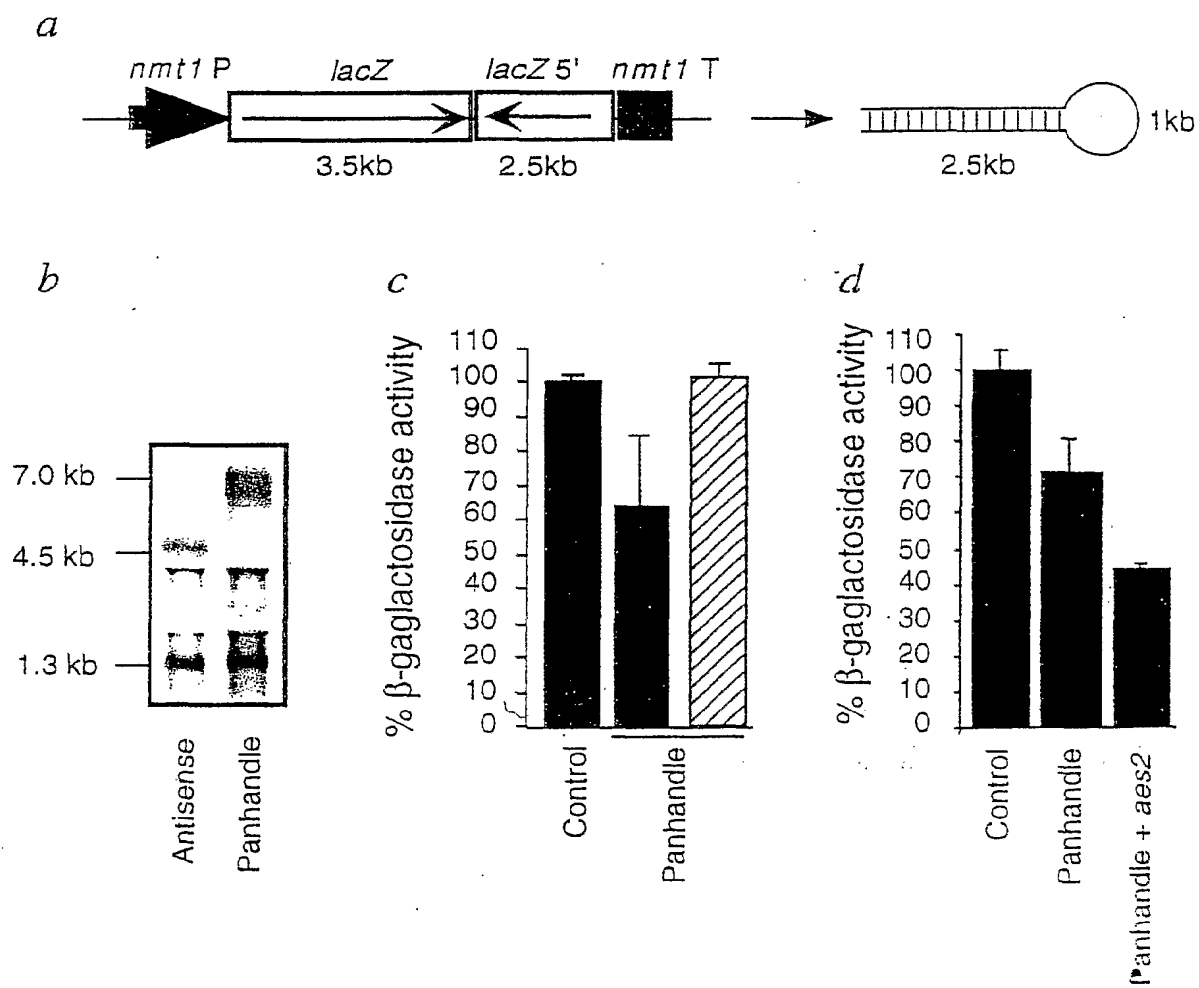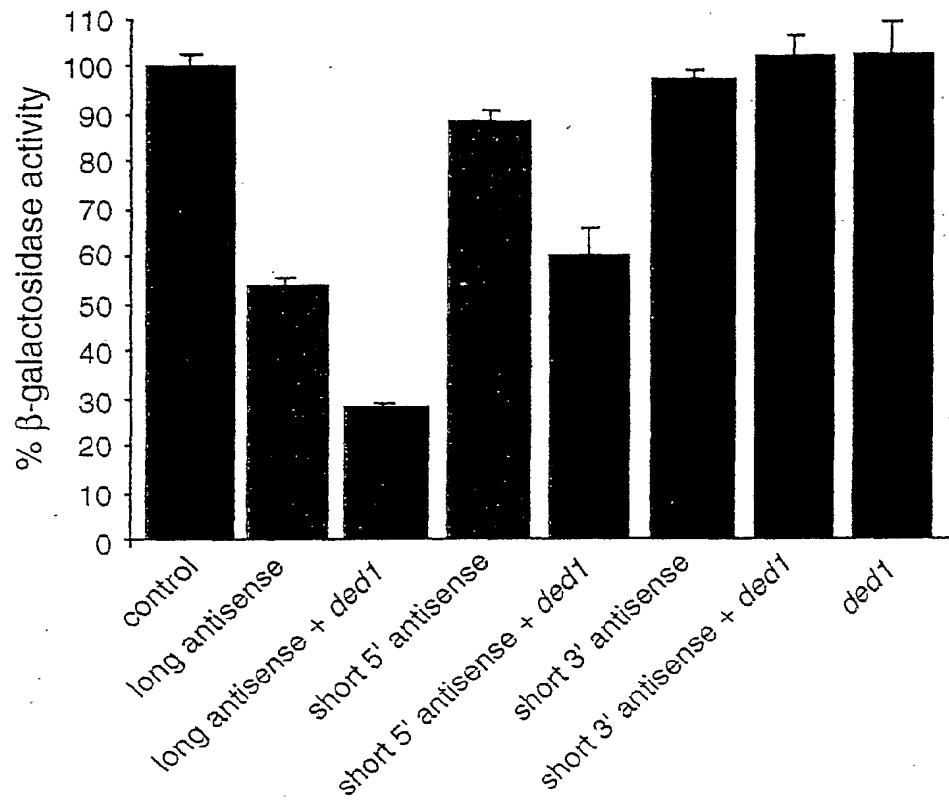

Figure 9.

Figure 10

Figure 11

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